PROTEOGLYCANS OF ADULT HUMAN GINGIVAE

by

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SUMMARY

The proteoglycans of adult human gingival epithelium and connective tissue have been studied. Essentially two main approaches were adopted:

(a) The isolation, identification and characterization of the glycosaminoglycan and protein components of the proteoglycans.

(b) Characterization of the intact proteoglycan molecules.

Following enzymatic digestion of separated gingival epithelium and connective tissue, the uronic acid content of these two tissue types amounted to 0.07% and 0.23% of their dry weights respectively. Four glycosaminoglycan species which corresponded to hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 were detected by electrophoresis. Confirmation of the molecular species present was obtained by specific enzyme or chemical degradation of the glycosaminoglycans. Neither chondroitin sulphate 6 nor keratan sulphate were observed.

There were quantitative differences noted in the distribution of the various glycosaminoglycans between epithelium and connective tissue. Heparan sulphate appeared to constitute the greatest proportion of the epithelial glycosaminoglycans (59.1%), whilst dermatan sulphate was the predominant connective tissue glycosaminoglycan (60.6%).

The number-average molecular weights of human gingival epithelium and connective tissue sulphated glycosaminoglycans were estimated by end group analysis. Data obtained for each sulphated glycosaminoglycan indicated values ranging from 15,000 for heparan

sulphate to 27,000 for dermatan sulphate. There appeared to be only slight inter-tissue differences between individual glycosaminoglycan species. The molecular weights of hyaluronic acid isolated from separated specimens of human gingival epithelium and connective tissue have also been estimated. The values were determined following substitution of sedimentation values obtained in an analytical ultracentrifuge into a previously determined empirical relationship between the reciprocal of the sedimentation coefficient at zero concentration and molecular weights estimated by sedimentation-diffusion. Values of molecular weight for connective tissue and standard hyaluronic acid were estimated at 340,000 and 205,000 respectively. Epithelial hyaluronic acid behaved in a more non-ideal fashion during ultracentrifugation than did the connective tissue and standard preparations. As a consequence, a range of molecular weights for epithelial hyaluronic acid between 860,000 and 372,000 was the best estimate possible.

Amino acid analyses of the protein cores of proteoglycans extracted from gingival epithelium and connective tissue under dissociative conditions (4 M guanidinium chloride) revealed profiles similar to other soft tissue proteoglycans. In both epithelium and connective tissue, glutamic acid was present in the greatest proportion (19.4% for the epithelium and 12.8% for the connective tissue). Aspartic acid and glycine were also major contributors to the overall amino acid profiles and there was little or no methionine detected. Overall, the epithelium was characterized by a relatively higher serine and glutamic acid content whilst the connective tissue

proteoglycans contained greater proportions of proline, leucine and arginine.

Proteoglycans extracted from human gingival epithelium and connective tissue under dissociative conditions were purified. The epithelial proteoglycans eluted as a single included peak on Sepharose 4B-CL and contained heparan sulphate and chondroitin sulphate 4 glycosaminoglycans. The connective tissue proteoglycans separated into three major populations on Sepharose 4B-CL. Of these populations, only the largest sized material was excluded from this gel under associative conditions (0.5 M sodium acetate). Following subsequent fractionation of the excluded material under dissociative conditions (4 M guanidinium chloride) there appeared to be an absence of aggregate formation of molecules within this population. The connective tissue proteoglycans contained heparan sulphate, dermatan sulphate and chondroitin sulphate 4, the proportions of which varied with the molecular size of the proteoglycans.

These results highlight various differences between the proteoglycans in human gingival epithelium and connective tissue and are thought to reflect both structural and physiological features unique to each of these two closely apposed tissues.

DECLARATION

This thesis contains no material which has been accepted or submitted for the award of any other degree or diploma in any University. Furthermore, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

The work described herein has been the subject of the following publications:

BARTOLD, P.M., WIEBKIN, O.W. and THONARD, J.C. (1981). Glycosaminoglycans of human gingival epithelium and connective tissue. Connective Tissue Research 9, 99-106.

BARTOLD, P.M., WIEBKIN, O.W. and THONARD, J.C. (1982). Molecular weight estimation of sulfated glycosaminoglycans in human gingivae. Connective Tissue Research 9, 165-172.

BARTOLD, P.M., WIEBKIN, O.W. and THONARD, J.C. (1982). Gingival epithelial and connective tissue proteoglycans. Biochemical Journal (In press).

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Interest in the biological properties of the extracellular components of connective tissues and epithelia has grown rapidly during the last fifteen years. In particular, the uronic acid containing, non-fibrous macromolecules of these matrices have been the subject of intense investigation. The intercellular components and their various interactions fulfil diverse functions in the animal body. Indeed, it has become increasingly apparent that they have physiological and pathological functions other than functioning solely as an inert supporting medium. Indeed, since all materials passing to and from cells must pass through these matrices, then, any changes in the state and composition of the extracellular macromolecules would be expected to exert a profound influence upon both the cells and the tissues which they comprise.

The extracellular matrix of connective tissue, disregarding structures such as blood vessels, bone, nerves etc. is comprised of structural fibres embedded within an extracellular ground substance. In contrast to connective tissue, epithelial tissue contains very little extracellular matrix because of the close apposition of the cells which constitute it. However, the small spaces that do exist between epithelial cells may contain material similar to the connective tissue ground substance. There are no fibrous components within epithelium.

As studies began to focus upon the non-fibrous elements within these matrices, a nomenclature evolved to describe the different

types of components noted. The term mucopolysaccharide was introduced by MEYER (1938) to describe "*...hexosamine containing polysaccharides of animal origin either in a pure state or as protein salts.*" The prefix, muco, was chosen to denote the relationship of this type of substance with mucous, the physiologic term for a viscous secretion. Although the mucopolysaccharides were found to be complexed with protein or peptide residues, the polysaccharide alone could be extracted or isolated without undergoing any appreciable degradation. Subsequent classifications of the mucopolysaccharides have been limited to the types of hexosamine and uronic acid which constitute these carbohydrates as well as to the presence or absence of sulphate residues.

Today, these polysaccharides are referred to as glycosaminoglycans and are considered to be ubiquitous components of all extracellular matrices. The term mucopolysaccharide may be still found in the current literature but is generally used to refer to the localization of these materials by histochemistry. A universally accepted terminology for the glycosaminoglycans was contrived by JEANLOZ (1960) and has replaced the old terminology associated with the mucopolysaccharides. A list of the old and corresponding new terms, together with the sugar and acidic residues which constitute these carbohydrates can be found in Table 1.1.

Generally, glycosaminoglycans rarely exist in a free state within tissues, rather, they are normally covalently bound to protein or peptide residues and accordingly are termed proteoglycans (LINDAHL and RODÉN, 1972; MATHEWS, 1975; MUIR and HARDINGHAM, 1975). Classically,

Table 1.1. Old and New Terms and Components of Glycosaminoglycans

Current Term	Old Term	Repeating Period Monosaccharides	Other Sugar Components	Acidic Group
Hyaluronic acid (Hyaluronate)	-	D-glucuronic acid D-glucosamine	-	Carboxyl
Chondroitin sulphate 4	Chondroitin sulphate A	D-glucuronic acid D-galactosamine	D-galactose D-xylose	Carboxyl Sulphate
Chondroitin sulphate 6	Chondroitin sulphate C	D-glucuronic acid D-galactosamine	D-galactose D-xylose	Carboxyl Sulphate
Dermatan sulphate	Chondroitin sulphate B	D-glucuronic acid L-iduronic acid D-galactosamine	D-galactose D-xylose	Carboxyl Sulphate
Heparan sulphate	Heparatin sulphate	D-glucuronic acid L-iduronic acid D-glucosamine	D-galactose D-xylose	Carboxyl Sulphate
Heparin	-	D-glucuronic acid L-iduronic acid D-glucosamine	D-galactose D-xylose	Carboxyl Sulphate
Keratan sulphate	Keratosulphate	D-galactose D-glucosamine	D-galactosamine D-mannose L-fucose Sialic acid	Sulphate

the structure of proteoglycans has been described as a single protein chain or core, to which many unbranched glycosaminoglycan chains are covalently bound in a "bottle brush" arrangement. A more detailed discussion of glycosaminoglycans and proteoglycans is presented later in this chapter.

Proteoglycans extracted from cartilage were the first to be seriously studied and are by far the most characterized species. Consequently, a great deal of information has accrued concerning the structure and function of these proteoglycans. As researchers began to investigate the compositions of other connective tissue matrices, the structure of the ground substances, proteoglycans in particular, was considered to be similar because glycosaminoglycan species resembling those found in cartilage were identified in other tissues. However, it is now recognized that cartilage proteoglycans have properties peculiar to that tissue. Furthermore, the glycosaminoglycan species of cartilage, whilst similar to other glycosaminoglycans, are limited to only keratan sulphate, chondroitin sulphate and hyaluronic acid. Hence, generalizations between the constituents of tissues of different origin must be qualified.

Nonetheless, such investigations on cartilage, as well as many other connective tissue proteoglycans, have provided a sound background knowledge for the concepts of this thesis.

ENGEL (1953), demonstrated the presence of water soluble mucoproteins in gingivae. Since then, other early literature has cited histochemical demonstrations of mucopolysaccharides within the ground substance of gingival tissue (DEWAR, 1955; SCHULTZ-HAUDT, 1957; THONARD and SCHERP, 1962; SCHULTZ-HAUDT, FROM and NORDBÖ, 1964;

TOTO and GRANDEL, 1966). The histochemical criteria used for these identifications were based upon various dye affinities for the acid mucopolysaccharides. Further studies demonstrated improved specificity for these extracellular components following substrate elimination by pretreatment of histological sections with specific enzymes (QUINTARELLI, 1960 a & b; THONARD and SCHERP, 1962; COHEN, 1968).

More recently, biochemical analyses on whole gingival tissue (epithelium and connective tissue) from various species have produced a variety of data concerning the precise identification of the constituent glycosaminoglycans. For example, components identified as glycosaminoglycans isolated from human gingivae were reported by CIANCIO and MATHER (1971) to contain only chondroitin sulphate 4 and chondroitin sulphate 6. However, HIRAMATSU, ABE and MINAMI (1978), using porcine gingivae, could demonstrate only chondroitin sulphate 4 together with dermatan sulphate, hyaluronic acid and heparan sulphate but not keratan sulphate or chondroitin sulphate 6. The absence of chondroitin sulphate 6 was also noted by SAKAMOTO, OKAMOTO and OKUBA (1978). In addition, EMBERY, OLIVER and STANBURY (1979) have demonstrated the presence of hyaluronic acid, dermatan sulphate and only chondroitin sulphate 4 in whole human gingivae.

Whilst the presence of glycosaminoglycans in gingival connective tissue is unequivocal, the presence of these molecules in gingival epithelium has been disputed (PEDLAR, 1979). Indeed, for many years it was assumed that gingival epithelium was not capable of synthesizing mucopolysaccharides and any histochemical observations of these components within the extracellular matrix of epithelium indicated an origin from the underlying connective tissue cells (BRAUN-FAULCO, 1958).

At present, the only information relating to this is derived from histochemical studies (THONARD and SCHERP, 1959 and 1962; THILANDER, 1963; CIMANSONI, FIORRE-DONNO and HELD, 1963; CIMANSONI and HELD, 1964; COHEN, 1968) and autoradiographic studies (TOTO and GRANDEL, 1966). Recent autoradiographic studies (WIEBKIN, et al., 1980; WIEBKIN and THONARD, 1981) and electronmicroscopy (LISTGARTEN, 1964; SCHROEDER and THIELADE, 1966; MELCHER and CHAN, 1978) have revealed further identification of materials interpreted as glycosaminoglycans within the extracellular "spaces" of human gingival epithelium. Nevertheless, PEDLAR (1979), interpreting his histochemical findings, concluded that the intercellular substance of gingival epithelium does not contain glycosaminoglycans.

With respect to proteoglycans in gingiva, very little has been published and most studies have focused on the glycosaminoglycan composition of gingival tissues as a whole with little consideration for the intact proteoglycan molecule. DZIEWIATKOWSKI, LAVALLEY and LAVALLEY (1977) reported, in abstract form, the presence of a proteoglycan in bovine gingiva which promoted fibrillogenesis of foetal calf skin salt soluble collagen and inhibited fibrillogenesis of acid soluble collagen from the same source. EMBERY, OLIVER and STANBURY (1979) studied the proteoglycan composition of normal and inflamed human gingival tissue and reported catabolism of the protein moiety of the proteoglycan leading to a loss of molecular structural integrity. More recently, a report by TOMIOKA (1981) described the extraction and purification of a dermatan sulphate proteoglycan from bovine gingiva.

AIM OF INVESTIGATION

Since the literature regarding the glycosaminoglycan composition of human gingival epithelium and connective tissue is equivocal and there is very little data concerning the proteoglycans of gingivae as a whole, the aim of this study was to carry out a comparative study of the proteoglycans of adult human gingival epithelium and connective tissue. This tissue was assumed to represent the closest approximation to healthy gingival tissue since it was obtained by gingivectomy on patients who had been subjected to pre-surgical gingival curettage and whose gingiva demonstrated no overt signs of clinical inflammation.

The characteristics studied more particularly were (a) the glycosaminoglycan compositions of the proteoglycans of each tissue, (b) the molecular weights of the constituent glycosaminoglycans, (c) the amino acid composition of the proteoglycan core proteins, and (d) the general nature of intact proteoglycan molecules.

The acquisition of such information is central to our understanding of the molecular biology of the extracellular matrix of gingivae. Indeed, it is hoped that the results obtained from this project will provide base-line data to which proteoglycans from inflamed gingivae affected by periodontal disease may be compared at a later date.

LITERATURE REVIEW

This review is not intended to be a comprehensive overview of the proteoglycans. Rather, emphasis will be placed upon those reports in the literature which form the nucleus of our present knowledge of proteoglycans, together with pin pointing a variety of recent investigations concerned with the finer details of these complex macromolecules.

Consideration will be first given to the structural components of proteoglycans followed by a discussion of various proteoglycan interactions and proteoglycan degradation. At this stage, it must be pointed out that only those glycosaminoglycans similar to those present in human gingiva as demonstrated by the work in this thesis will be discussed. More detailed information concerning all seven known mammalian glycosaminoglycans and the proteoglycans in general may be found in the following extensive recent reviews: LINDAHL and HÖÖK (1978); COMPER and LAURENT (1978); KENNEDY (1979); CHAKRABARTI and PARK (1980).

GENERAL PROTEOGLYCAN STRUCTURE

The term "proteoglycan" was first introduced in 1967 (see BALAZS, 1973) to describe a family of macromolecules comprising many glycosaminoglycan chains covalently bound to a single protein core. Previously, these molecules had been referred to as "protein-polysaccharide complexes" or "chondromucoprotein".

MATHEWS and LOZAITYTE (1958) were the first to propose a structure for the protein-polysaccharide complexes extractable from cartilage. They suggested that the molecules existed, in solution, as a rod shaped particle of length 3,500 Å which included approximately 60 flexible chondroitin sulphate chains. Shortly after this model was presented, PARTRIDGE, DAVIS and ADAIR (1961) proposed a similar

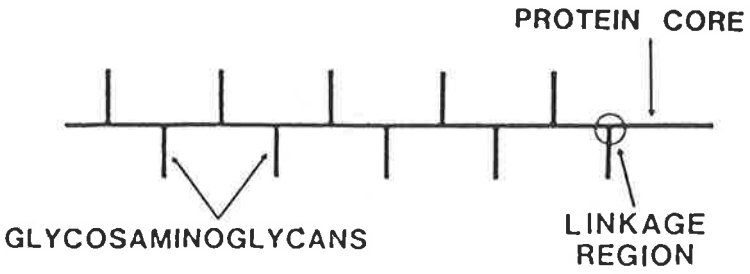
arrangement of protein and carbohydrate for the protein-polysaccharide complexes of cartilage.

These early concepts of a single protein core to which many side chains of glycosaminoglycans are bound are still essentially correct and may be taken as the elementary model for most proteoglycans (excluding hyaluronic acid). Such an arrangement is schematically represented in Figure 1.1. This model is, however, oversimplistic. Proteoglycans may be comprised of either only one type (species) of glycosaminoglycan (FRANSSON and RODÉN, 1967 a; ÖBRINK, 1972; DAMLE, et al., 1979; DAMLE, COSTER and GREGORY, 1982) or they may contain several types of glycosaminoglycans on the same protein core (TSIGANOS and MUIR, 1969; FRANSSON and HAVSMARK, 1970; EISENSTEIN, et al., 1975). Furthermore, smaller oligosaccharides have been shown also to be integral components of some proteoglycans (THONAR and SWEET, 1977; DE LUCA, et al., 1980; LOHMANDER, et al., 1980).

Therefore, proteoglycans are comprised of (a) protein core (b) glycosaminoglycans and (c) oligosaccharides. These components have been well studied for cartilage proteoglycans and are listed in Table 1.2. The values seen in Table 1.2 are based on data obtained by HASCALL and SAJDERA (1970) and HASCALL and RIOLO (1972).

The size of the core protein of proteoglycans from bovine nasal cartilage was computed from the molecular weight of both the proteoglycan molecule and its constituent glycosaminoglycan chains. On the presumption that these molecules contain 7% protein, a value of 2.5×10^5 was derived. Similarly, it was calculated that up to 150 chains of glycosaminoglycans could be attatched to a single protein core of the above dimensions.

a. PROTEOGLYCAN



b. LINKAGE REGION SEQUENCE

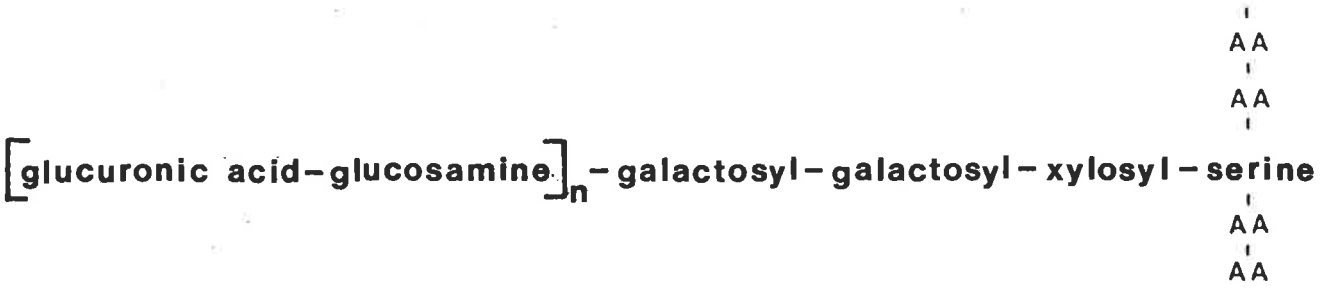


Figure 1.1.

Schematic representation of:

(a) Proteoglycan molecule

(b) Linkage region of glycosaminoglycans to protein core.

Table 1.2

Components of Cartilage Proteoglycans

Composition	No. of Chains	Mol.Wt.	Percentage of total weight
Protein	1	$2 \times 10^5 - 3 \times 10^5$	7-12
Chondroitin Sulphate	100	20000	80-85
Keratan Sulphate	50	5500	7
Oligosaccharides	50	1200-2000	1-3

Charged groups

Sulphate	4500 per molecule
Carboxylate	4200 per molecule
Total	8700 per molecule

The data in this table was taken from HARDINGHAM (1981) and is essentially based on that of HASCALL and SAJDERA (1970) and HASCALL and RIOLO (1972).

GLYCOSAMINOGLYCANS

Glycosaminoglycans are the major carbohydrate components of proteoglycans. Structurally, they are characterized by repeating disaccharide units of an uronic acid moiety, either D-glucuronic acid or L-iduronic acid, and an hexosamine moiety, either D-glucosamine or D-galactosamine. Keratan sulphate is an exception to this generalization since it contains D-galactose in place of uronic acid (see Table 1.1). Hence the term glycosaminoglycan is derived from its structural components: glycosamine (amino sugar) and glycans (polysaccharides).

With the exception of hyaluronic acid, the glycosaminoglycans contain sulphate groups, which, together with carboxyl groups present on all glycosaminoglycan species (Table 1.1), form a negatively charged molecule under physiological conditions. As it is these highly negatively charged chains which are responsible for much of the overall configuration of proteoglycans in vivo, it is appropriate to consider them first.

HYALURONIC ACID

Hyaluronic acid was first isolated by MEYER and PALMER (1934) from the vitreous humor of cattle eyes. Since then it has been described as an ubiquitous extracellular macromolecule (TOOLE, 1973), being present in such diverse sites as the coating of oocytes, blood vessels, skeletal tissues, brain and umbilical cord.

Structurally, hyaluronic acid is the simplest of the glycosaminoglycans. The detailed structure of this polysaccharide was established by WEISSMANN and MEYER (1954). The repeating dissaccharide units are β-D-glucuronic acid and 2-acetamido-2-deoxy-β-D-glucose. Both of these units are in the pyranose form.

Apart from the absence of sulphate groups, one of the many ways in which hyaluronic acid may be distinguished from the other glycosaminoglycans

is by its susceptibility or not to various enzyme treatments (as reviewed by LINKER and HOVINGH, 1977; MURATA, 1980). It is resistant to degradation by both chondroitinase AC II (from Flavobacterium heparinium) and by chondroitinase ABC (from Proteus vulgaris). On the other hand, hyaluronic acid is degraded by hyaluronidases from microbial and animal origins.

Reported values for the molecular weights of hyaluronic acid range from 10^4 to 10^7 depending upon the source of its extraction, the extraction procedures utilized and the type of techniques used to estimate or measure molecular weight. Nonetheless, despite such a wide range, hyaluronic acid has by far the highest molecular weight of the glycosaminoglycans (VARGA, 1955; NICHOL, OGSTON and PRESTON, 1967; SWANN, 1969 a; CLELAND and WANG, 1970; SHIMADA and MATSUMARA, 1975). Further considerations of the molecular weight of hyaluronic acid and its measurement may be found in Chapter 5.

Many of the physical characteristics of hyaluronic acid from various tissues have been well documented (BALAZS, 1958; ROGERS, 1961; SUNDBLAD, 1965; LAURENT, 1970). For example, it has been postulated that this molecule acts as a biological absorbant to mechanical stress, thereby protecting the cells from compressional and other functional or abusive forces. In addition, hyaluronic acid has an exceptionally high affinity for water and, as a result, is responsible for maintaining the hydration of most tissues (COMPER and LAURENT, 1978). Indeed, it is inviting to postulate that hyaluronic acid could be responsible for the rehydration of tissues following the expulsion of water and other tissue fluids from areas subjected to compressional forces; this has not been

demonstrated. Hyaluronic acid may also serve as a lubricating agent at the interface between solid surfaces such as tendons, tendon sheaths and synovial membranes and their surrounding fluid matrices.

Clinically, hyaluronic acid has been used during intraocular surgery as a substitute for aqueous humor in diseases of the corneal epithelium (BALAZS and GIBBS, 1970; MILLER, O'CONNOR and WILLIAMS, 1977) as well as facilitating intraocular lens implantations, corneal transplantations and cataract extraction (PAPE and BALAZS, 1980).

Hyaluronic acid has been used also, in the treatment of inflamed or traumatized joints (RYDELL, BUTLER and BALAZS, 1970; ÅSHEIM and LINDBLAD, 1976). However, its pharmaceutical action in these treatments appears not to be one of lubrication but rather one of impairing the formation of scar tissue between the damaged joint surface and its surrounding tissues (BALAZS and DARYNZIEWCZ, 1973). Such control of cell responses, has been ascribed to the interaction of hyaluronic acid with cell surface receptors (BALAZS, 1977).

Evidence suggesting that hyaluronic acid influences cellular activity was first advanced by TOOLE (1973) who demonstrated that hyaluronic acid is enriched during the early stages of the development of tissues such as chick embryo limbs and cornea and regenerating or remodelling tissues such as new limbs. He further suggested that hyaluronic acid inhibits chick somite cell interactions during development, thereby permitting migration and proliferation of these cells but delaying the onset of differentiation until a correct sequence of tissue organization could ensue. Additional evidence for the influence of hyaluronic acid on cellular activity, is its

inhibitory effect on the synthesis and secretion of proteoglycans by cultured adult chondrocytes (WIEBKIN and MUIR, 1973 a & b; SOLURSH, VAERWYCK and REITER, 1974). Such inhibition was proposed to be effected through the interaction of hyaluronic acid with binding sites on the cell surfaces, which may be proteoglycans.

The covalent association of protein with sulphated glycosaminoglycans to form proteoglycans is well documented and is discussed in detail later. However, such covalent association of hyaluronic acid with protein is not as clear and will therefore be discussed now.

OGSTON and STANIER (1950) demonstrated that hyaluronic acid, prepared and purified from synovial fluid by mild procedures, contained up to 20% protein. Subsequently, it was shown that most of this protein could be removed by mild physical treatments (PRESTON, DAVIES and OGSTON 1965). Nonetheless, hyaluronic acid prepared from a variety of sources is associated with protein to varying degrees. Such discrepencies are related to both the conditions used for extraction as well as to the original source of the hyaluronic acid (NICHOL, OGSTON and PRESTON, 1967; SWANN, 1968 a; SILPANATA, DUNSTONE and OGSTON, 1968). A more detailed study of the protein component of hyaluronic acid was reported by SWANN (1968 b), in which the amino acids, serine, glycine, aspartic acid and glutamic acid accounted for 65% of the total amino acids associated with hyaluronic acid isolated from rooster comb. The overall contribution of such protein components to the hyaluronic acid-protein complex was approximately 0.33% of the total weight. Treatment of these complexes with alkali to remove the protein resulted in a decrease in the serine content but no apparent reduction in the molecular size of hyaluronic acid. This implied that either β-elimination reactions did not occur, or if they did, only a

limited number of amino acids were associated with hyaluronic acid in a mode which allowed removal by β-elimination (e.g. glycopeptide linkages involving serine). Further studies on proteins associated with synovial fluid hyaluronic acid have indicated them to be mainly α-globulin with some β-lipoproteins; albumin and γ-globulin have also been implicated in contributing to such protein-polysaccharide complexes (SILPANATA, DUNSTONE and OGSTON, 1969; FRASER et al., 1977). Such interactions vary greatly with changes in pH, ionic strength as well as hyaluronic acid concentration.

Subsequent characterizations of hyaluronic acid-protein complexes and final proof of covalent linkage similar to that of proteoglycans have proven extremely difficult in view of the nature of hyaluronic acid and the small amounts of protein associated with it (BAXTER and FRASER, 1980).

CHONDROITIN SULPHATE 4 and 6

Chondroitin sulphate was first isolated by KRUKENBERG (1884) and later by MÖRNER in 1889. Much later, MEYER et al., (1956), reported the presence of three structural isomers of chondroitin sulphate. These were originally termed chondroitin sulphate A, B and C but subsequently renamed chondroitin sulphate 4, dermatan sulphate and chondroitin sulphate 6 respectively (see Table 1.1).

Since the structures of chondroitin sulphates 4 and 6 differ only in the position of their sulphate group, it is appropriate to consider them under one heading. However, dermatan sulphate is sufficiently different to allow it to be considered separately.

The distribution of chondroitin sulphate 4 and 6, like hyaluronic acid, is widespread throughout the connective tissues of mammals. However, whilst both isomers may be found in the same tissues (e.g. skin, aorta and cartilage), chondroitin sulphate 4 is more

characteristic of foetal tissues (MATHEWS, 1965 a). For example the ratio of chondroitin sulphate 4 to chondroitin sulphate 6 in articular cartilage decreases with age such that healthy, mature cartilage contains almost exclusively chondroitin sulphate 6 (MURATA and BJELLE, 1976; MURATA and BJELLE, 1979; MICHELACCI et al., 1976).

Structurally, the functional units of chondroitin sulphate 4 and 6 consist of O-sulphated 2-acetamido-2-deoxy-β-D-glucose linked through a 1-4 glycosidic bond to β-D-glucuronic acid. Hence, both yield the same dissachariде unit, chondrosine, upon hydrolysis. As a result, these two glycosaminoglycans originally could be distinguished only by their optical rotations or by the solubility of their calcium salts in aqueous ethanol (MEYER and PALMER, 1936). However, subsequent investigations revealed positional differences in the sulphate ester group on the hexosamine residues between chondroitin sulphates 4 and 6; chondroitin sulphate 4 being O-sulphated at position C-4 whereas chondroitin sulphate 6 was O-sulphated at C-6 of the hexosamine.

These isomers may also be distinguished following enzymatic digestions by chondroitinase AC II (from Flavobacterium heparinium) or chondroitinase ABC (from Proteus vulgaris) either of which will liberate the respective disaccharide fragments of chondroitin sulphate 4 and 6 (SAITO, YAMAGATA and SUZUKI, 1968).

Despite the apparent simple structure of these two glycosaminoglycan isomers, the above descriptions are oversimplified.

Indeed, there is a spectrum of forms in which the chondroitin sulphates may exist (MATHEWS and DECKER, 1968 a). For example, BETTELHEIM and PHILPOTT (1960) and BETTELHEIM (1964) isolated partially sulphated species of chondroitin sulphate 4 from bovine tracheal cartilage. Undersulphated chondroitin sulphate 4 has also been reportedly extracted from human plasma and rat rib (JUVANI, et al., 1975; LIAU, GALICKI

and HOROWITZ, 1978). On the other hand, an over sulphated species of chondroitin sulphate has been detected in human ear skin (BARKER, KENNEDY and CRUIKSHANK, 1969) and bone (HJERPE, et al., 1982). Further studies have indicated that the relative distribution of sulphate residues may vary along certain sections of the chondroitin sulphate chains. Relatively little sulphate has been identified in the vicinity of the carbohydrate-peptide linkage region, whilst greater amounts have been detected along the peripheral portions of the polysaccharide chain (WASTESON and LINDAHL, 1971).

Using osmotic pressure and light scattering methods, MATHEWS (1959) reported molecular weights in the order of 1.7 to 5.0 x 10^4 for both chondroitin sulphate 4 and 6 glycosaminoglycan chains extracted from bovine nasal septum and human chordoma respectively. Since then, various reports concerning the distribution of molecular weights for chondroitin sulphate chains have appeared in the literature with one suggesting a range as wide as 0.8 x 10^4 to 4.3 x 10^4 (YAMAGUCHI, 1980). Gel chromatographic methods of molecular weight estimation have also demonstrated a range of molecular weights for chondroitin sulphate chains (WASTESON, 1969; ROBINSON and HOPWOOD, 1973). In these instances, the distribution of molecular weight or molecular size for a population of chondroitin sulphate chains isolated from nasal septum appeared "bimodal". Such results suggested that two species of chondroitin sulphate with respect to molecular weight were present in this tissue (HOPWOOD and ROBINSON, 1973). Consequently, estimates of the average molecular weight of chondroitin sulphate from nasal cartilage have been placed in the order of 1.35 to 2.5 x 10^4 (ROBINSON and HOPWOOD, 1973).

Similarly, studies concerning the molecular weight of chondroitin sulphate proteoglycans have produced a variety of values. Molecular weight estimations for chondroitin sulphate proteoglycans from cartilage (or protein-polysaccharides as they were originally termed) appeared as early as 1958. Such early reports suggested molecular weights for these complexes ranging from several million (MATHEWS and LOZAITYTE, 1958; LUSCOMBE and PHELPS, 1967 a & b) to 2.4×10^5 (PARTRIDGE, 1966; MUIR and JACOBS, 1967). However, the techniques available at this time made it difficult to obtain material free from contaminating agents, and, on the basis of present knowledge, such extractions most certainly would have contained keratan sulphate and extraneous protein. Thus, these molecular weight values did not truly represent a value for chondroitin sulphate proteoglycan, but they did provide useful information regarding the polydispersity of such extractable protein-polysaccharide components of cartilage.

Indeed, proteoglycans containing chondroitin sulphate generally possess other glycosaminoglycan species. For example, cartilage proteoglycans are comprised of both chondroitin sulphate and keratan sulphate (HOFFMAN, MASHBURN and MEYER, 1967) whilst skin and aortae proteoglycans appear to contain chondroitin sulphate and dermatan sulphate (ÖBRINK, 1972; RADHAKRISHNAMURTHY, RUIZ and BERENSON, 1977). Therefore, most reported molecular weight values for chondroitin sulphate proteoglycan are likely to represent a value for chondroitin sulphate and other constituent glycosaminoglycans comprising the proteoglycan in question. Recently, a proteoglycan reported to contain exclusively chondroitin sulphate was isolated from porcine skin (DAMLE, et al., 1982). A tentative model describing a molecule

with a mass of 10^6 daltons containing 50 chains of chondroitin sulphate was presented. However, despite very careful preparation and purification of their material, some glucosamine was identified (8% of the total hexosamine). A likely source of this sugar residue could have been heparan sulphate, which indicates the likelihood of this material being a hybrid heparan sulphate-dermatan sulphate proteoglycan.

DERMATAN SULPHATE

Dermatan sulphate, first isolated from pig skin by MEYER and CHAFFEE in 1941, has since been identified widely throughout the connective tissues of mammals (MEYER, et al., 1956). At this time, dermatan sulphate was considered to be an isomer of chondroitin sulphate, differing structurally from chondroitin sulphates 4 and 6 by the presence of α-L-iduronic acid rather than β-D-glucuronic acid.

Although small portions of D-glucuronic acid and D-glucose have been detected in the hydrolysates of dermatan sulphate (HOFFMAN, LINKER and MEYER, 1956; STOFFYN and JEANLOZ, 1960), the possibility of contamination of these preparations by chondroitin sulphate due to the limited specificity of the glycosaminoglycan separation/purification techniques available at this time cannot be discounted. Nonetheless, subsequent analyses on dermatan sulphates prepared by more sensitive purification procedures have confirmed the presence of varying proportions of D-glucuronic acid. This was deduced from the observation that some susceptibility of this glycosaminoglycan to testicular hyaluronidase could be demonstrated (FRANSSON and RODÉN, 1967 a & b; FRANSSON, 1970). The observations of these, and others (HABUCHI, et al., 1973; MICHELACCI and DIETRICH, 1975) of structural microheterogeneity therefore imply that both β-D-glucuronic acid as well as α-L-iduronic acid can be integral units of dermatan sulphates.

The position and configuration of the glycosydic linkages are similar to those of the chondroitin sulphates, namely, a 1-4 glycosidic bond between the uronic acid residue and N-acetyl galactosamine sulphate. The ratio of sulphate per disaccharide unit is greater than unity (SUZUKI, et al., 1968). For example, dermatan sulphate disaccharides may be disulphated, with the sulphate groups being located on the galactosamine group at 0-4 or 0-6 (FRANSSON, 1968 a; HAMER and PERLIN, 1976). In addition, sulphate groups may also be found on the L-iduronic acid residues at position 0-2 (MALMSTRÖM and FRANSSON, 1971; FRANSSON, et al., 1974).

The overall structural arrangement of both L-iduronic acid and D-glucuronic acid residues within dermatan sulphate chains is assumed to be in alternating groups or clusters at variable intervals along the chain (FRANSSON and RODÉN, 1967 b; FRANSSON, 1968 a; FRANSSON and HAVSMARK, 1970; MICHELACCI and DIETRICH, 1975). More recently, detailed structural analyses of dermatan sulphate have been complicated by the identification of two different species: an aggregating form and a non-aggregating form (FRANSSON, 1976; CÖSTER and FRANSSON, 1977; FRANSSON and CÖSTER, 1979).

Whilst both the aggregating and non-aggregating chains contain similar proportions of iduronic acid-glucosamine and glucuronic acid -glucosamine units, the spatial arrangement of these repeating units within the chains differs markedly. Both types contain segments containing either only iduronic acid-glucosamine units or only glucuronic acid-glucosamine units; however regions with these two disaccharide units mixed together (void of periodicity of segments containing only iduronic acid-glucosamine units or only glucuronic acid-glucosamine units) are present almost exclusively in the aggregating chains. Repeating units

containing iduronic acid are higher in the non-aggregating chains (FRANSSON and CÖSTER, 1979). On the basis of these observations, it was proposed that aggregating dermatan sulphate chains exclusively contained segments of [-(-glucuronic acid-hexosamine)$_m$-(iduronic acid-hexosamine)$_n$-] . Where m and n =1 as well as larger segments where m and n were greater than 1. On the other hand, non-aggregating species were comprised of only segments where m and n were always greater than 1.

Reports concerning the molecular weights of dermatan sulphate chains from a variety of tissues are scant. However, the reports of those who have attempted to determine such a value for dermatan sulphate, indicate that the molecular weights are polydisperse, ranging from 1.7 to 5.0 x 10^4 (HILBORN and ANASTASSIADES, 1971; WASTESON, 1971; RADHAKRISHNAMURTHY, et al., 1980). This range of values is very similar to that reported for chondroitin sulphate 4 and 6 (see earlier section, this chapter).

Dermatan sulphate in the form of a proteoglycan was first reported by TOOLE and LOWTHER (1968) and subsequently demonstrated by FRANSSON (1968 b) to have an identical carbohydrate-protein linkage region to chondroitin sulphate 4. Nonetheless, dermatan sulphate proteoglycans have proven difficult to study because of the difficulty in extracting them from tissues as "pure" dermatan sulphate proteoglycans. Indeed, these proteoglycans usually exist as hybrids of both dermatan sulphate and chondroitin sulphate chains on the same protein core (KRESSE, HEIDEL and BUDDECKE, 1971; EISENSTEIN, et al., 1975; RADHAKRISHNAMURTHY, RUIZ and BERENSON, 1977).

Dermatan sulphate proteoglycans claimed to be devoid of other glycosaminoglycans have, nonetheless, been extracted from pig skin (ÖBRINK, 1972; DAMLE, et al., 1979). These have been demonstrated to

contain between 58-60% protein and were polydisperse with respect molecular weights, having an average molecular weight value of 2.9×10^6. A "pure" dermatan sulphate proteoglycan has also been extracted from bovine sclera (SHEEHAN, et al., 1981; COSTER and FRANSSON, 1981), which could be fractionated into one larger and one smaller pool which differed in amino acid compositions as well as relative amounts of glucuronic acid and iduronic acid.

HEPARAN SULPHATE

Amongst the glycosaminoglycans, heparan sulphate has been the least studied, receiving little attention until recent years. This polysaccharide was first isolated from lung and liver in 1948 (JORPES and GARDELL) and is not necessarily considered to be a discrete glycosaminoglycan species, but rather represents part of a spectrum of similar polysaccharides. This heterogenous group has heparin at one end and heparan sulphate at the other. The glycosaminoglycans which constitute this spectrum are thus often termed "heparin-like" polysaccharides.

Heparan sulphate is essentially found extracellularly, being widely distributed in most mammalian tissues (LINKER and HOVINGH, 1973; DIETRICH, et al., 1977; TOLEDO and DIETRICH, 1977). More precisely, heparan sulphate is located in the microenvironment of cells and is closely associated with cell surfaces (DIETRICH and DE OCA, 1970; KRAEMER, 1971 a & b; ROBLIN, et al., 1975; OLDBERG, et al., 1977; DIETRICH and DE OCA, 1978; GLIMELIUS, et al., 1978). In addition, recent studies have demonstrated that heparan sulphate is secreted into the extracellular matrix produced by fibroblast-like cells in vitro (HEDMAN, et al., 1979; ROLLINS and CULP, 1979). Thus, heparan sulphate appears to occur in two distinct forms: one associated

with cellular membranes and the other with the extracellular matrix (NORLING, GLIMELIUS and WASTESON, 1981).

Structurally related to heparin, heparan sulphates comprise a group of related molecules based on a backbone of alternating glucosamine and hexuronic acid joined via β 1-4 linkages, but differing in sulphate content and in arrangement of charged groups along this backbone (CIFONELLI and DORFMAN, 1960; LINDAHL, 1970; LINKER and HOVINGH, 1973). Indeed, considerable structural heterogeneity is possible within this group of glycosaminoglycans (CIFONELLI, 1968; HOVINGH and LINKER, 1974). The glucuronic acid residues of heparan sulphate are either β-D-glucuronic acid or α-L-iduronic acid. The latter is usually sulphated at C-2, whilst the glucuronic acid components are invariably non-sulphated (LINDAHL, et al., 1977). The amino sugar moieties are either N-acetylated D-glucosamine, N-sulphated D-glucosamine or N-sulphated D-glucosamine which may also be sulphated at C-6. The degree of sulphation of N-sulphated glucosamine residues is less than that for the iduronic acid residues (CIFONELLI and KING, 1977), nonetheless, it is the presence of N-sulphated groups within the hexosamine moieties which distinguish heparan sulphate (and heparin) from other glycosaminoglycans.

More detailed information regarding the structure of heparan sulphate following combined enzymatic and chemical analyses has indicated that heparan sulphates are comprised of (a) uninterrupted blocks of N-acetyl-glucosamine-hexuronic acid repeating units, (b) larger blocks that contain N-sulphated glucosamine-hexuronic acid regions but lack extensive ester sulphation, and (c) segments composed of alternating N-sulphated glucosamine-hexuronic acid and N-acetylated glucosamine-hexuronic acid units together with some disulphated glucosamine moieties (LINKER and HOVINGH, 1975). Such variation

of N- and O- sulphation, which appears to occur in block segments along the polysaccharide chain, may depend on the degree of C-5 epimerization of the hexuronic acid residue during biosynthesis. Uronic acid epimerization and O-sulphation reactions are interrelated, whilst N-sulphation of the chain is concluded before O-sulphation (LINDAHL, et al., 1977).

Estimations of the glucuronic acid composition of heparan sulphates from a variety of tissues (lung, mucosa, umbilical cord, aorta) indicate that N-sulphated regions contain both iduronic acid and glucuronic acid residues, whereas N-acetylated regions contain glucuronic acid exclusively (TAYLOR, et al., 1973; HÖÖK, LINDAHL and IVERIUS, 1974). Furthermore, the ratios of N- and O-sulphate in these polymers generally increases with increasing iduronic acid content (TAYLOR, et al., 1973).

The molecular weights of heparan sulphate chains have been variously studied. Early claims that they were of dialysable size have since been refuted. Heparan sulphate isolated from human aorta was originally assigned a molecular weight of 2.3 to 2.9×10^4 (KNECHT, CIFONELLI and DORFMAN, 1967). However, later studies on similar preparations of heparan sulphate from human aorta placed their molecular weights at between 4 to 5×10^4 (ÖBRINK, et al., 1975; KLEINMAN, SILBERT and SILBERT, 1975). Larger molecular weight values have been reported for heparan sulphate chains from bovine aorta. These range in values up to a maximum of 1.7×10^5 (DIETRICH and NADER, 1974).

The proteoglycan nature of heparan sulphate has been confirmed from several tissues (JANSSON and LINDAHL, 1970; KRAEMER and SMITH,

1974). Heparan sulphate proteoglycans from rat liver membranes have been well characterized and appear to contain four polysaccharide chains attached to a protein core (KRAEMER and SMITH, 1974). Indeed, it seems that the proteoglycan nature of heparan sulphate is similar to other proteoglycans in that it contains the same glycopeptide linkage sequence that constitutes the polysaccharide-protein linkage region of other proteoglycans.

However, as already mentioned, detailed characterization of heparan sulphate proteoglycans has been complicated by the occurrence of two different types. One, which is intimately associated with cell surfaces, the other, specific for the extracellular matrix. Recently, the heparan sulphate proteoglycans of these two regions have been isolated and partially characterized (CARLSTEDT, COSTER and MALMSTRÖM, 1981). The proteoglycan associated with cell surfaces was of lower buoyant density and contained a higher proportion of protein compared to the matrix heparan sulphate proteoglycan. As a consequence of the higher proportion of protein in the cell surface associated heparan sulphate proteoglycan, it was proposed that this proteoglycan may be attatched to the cell membrane lipid bilayer via a hydrophobic anchor rich in hydrophobic amino acids such as leucine. This proposition has been confirmed subsequently by KJELLEN, PETTERSON and HÖÖK (1981) who demonstrated a cell surface heparan sulphate proteoglycan with its core protein embedded within the lipid bilayer of the plasma membrane of cells. KELLER, KRENTZ-LOWE and SAIDEL (1981) have also confirmed the association of heparan sulphate proteoglycans with cell surface membranes by demonstrating the presence of a hydrophobic portion of the proteoglycan protein core which is specifically associated with cell membranes.

THE GLYCOPEPTIDE LINKAGE

The nature of the region where glycosaminoglycan side chains and the protein core are linked has been extensively reviewed for proteoglycans of animal, plant and bacterial origin (LINDAHL and RODÉN, 1972). In all cases the reducing end of the terminal glycosaminoglycan monosaccharide appears to be covalently bound, via an O-glycosidic bond, to a side chain of an amino acid residue in the protein core.

MUIR (1958) first demonstrated that the only amino acid remaining with chondroitin sulphate 4 following extensive proteolytic digestion, was L-serine. Subsequent analyses of oligosaccharides released by acid hydrolysis of chondroitin sulphate 4-protein complexes, indicated the following carbohydrate sequence to be present in the glycopeptide linkage region (RODÉN and SMITH, 1966):-

$$\text{-[galactosamine-uronic acid]}_n\text{-galactosyl-galactosyl-xylosyl-O-serine}$$

In all instances, the glycopeptide linkage between xylose and serine was O-glycosidic (MUIR, 1958; BRENDEL and DAVIDSON, 1966; LINDAHL and RODÉN, 1966; RODÉN and SMITH, 1966).

An identical carbohydrate-protein linkage sequence to that described above for chondroitin sulphate 4 has been reported for chondroitin sulphate 6 (ANDERSON, HOFFMAN and MEYER, 1965).

Similarly, proteolytic digestion of dermatan sulphate-protein complexes demonstrated the predominace of L-serine, D-galactose and D-xylose in the residual carbohydrate fragments. This, therefore, indicated that the glycopeptide linkage for dermatan sulphate was identical to the chondroitin sulphates (MEYER, et al., 1965; RODÉN, 1965; BELLA and DANISHEFSKY, 1968; STERN, et al., 1969). More recently,

the linkage between dermatan sulphate and proteoglycan core protein has been confirmed to be O-glycosidic between xylose and serine by alkaline reduction of these proteoglycans (AKIYAMA and SENO, 1981).

Heparan sulphate has also been isolated from various tissues complexed with protein (KNECHT, CIFONELLI and DORFMAN, 1967) and later confirmed to be in a proteoglycan form (JANSSON and LINDAHL, 1970; RADHAKRISHNAMURTHY, RUIZ and BERENSON, 1977). Following proteolytic digestion of these proteoglycans, the polysaccharide chains were found to be linked to serine residues via a similar trisaccharide sequence as described above (KNECHT, CIFONELLI and DORFMAN, 1967; LINKER and HOVINGH, 1973).

To date, there is no general agreement whether hyaluronic acid is covalently bound to protein as a proteoglycan. This has already been discussed in detail previously (page 15). Nonetheless, the nature of the reported association of hyaluronic acid with protein will be discussed now.

One reason for the confusion regarding this subject is that hyaluronic acid can be extracted from tissues associated with protein. However, mild physical treatments (i.e. ultracentrifugation under associative conditions) removes a large proportion (but not 100%) of this associated protein, and therefore, most of the protein is obviously not covalently bound to hyaluronic acid. Nonetheless, glycopeptides associated with hyaluronic acid after purification have been studied. One such study reported the presence of D-galactose and D-glucose in hyaluronic acid glycopeptides prepared from synovial fluid (HAMERMAN, ROJKIND and SANDSON, 1966). Further studies on purified synovial fluid hyaluronic acid appeared to confirm that a

small proportion of protein is combined with this polysaccharide (SCHER and HAMERMAN, 1972). LAURENT (1970) reported the presence of minute amounts of protein associated with carefully purified rooster comb hyaluronic acid. The results of amino acid analysis of this protein component corresponded very closely to a similar hyaluronic acid-protein complex studied by SWANN (1968 b). The similarity of these two independent reports supports the concept of a "firmly bound" specific protein to hyaluronic acid. However, until the glycopeptide linkage region is isolated and characterized, confirmation of a covalent association between hyaluronic acid and protein is not possible.

PROTEIN STRUCTURE

Despite extensive investigations regarding the structural properties of glycosaminoglycans, only limited reports are available related to the structure of proteoglycan core protein.

The proportion of protein present in proteoglycans is variable between different types. For example, the typical cartilage chondroitin sulphate-keratan sulphate proteoglycan contains between 2% and 18% protein (HASCALL and SAJDERA, 1970) whilst dermatan sulphate proteoglycans from pig skin may contain up to 50% protein (ÖBRINK, 1972). Furthermore, the actual protein content may vary even for proteoglycans derived from the same source (HOFFMAN, et al., 1975). Such discrepancies in protein content may, therefore, reflect variability between different types of proteoglycan as well as their sensitivity to mechanical and proteolytic degradation during preparation.

The amino acid compositions of proteoglycan core proteins are characteristic for these macromolecules, in which serine, glycine,

proline and glutamic acid quantitatively predominate. However, whilst the amino acid sequence of the protein core is of great interest in understanding synthesis, carbohydrate branching points and possibly some biophysical properties of the proteoglycans, little information is available on such sequences. This is hardly surprising since the average size of a cartilage proteoglycan core protein has been estimated to be 200,000 daltons (HASCALL and RIOLO, 1972). To date, investigations regarding amino acid arrangement within proteoglycan core protein have focused on the link region between chondroitin sulphate and protein of proteoglycans extracted from cartilage. The polypeptide arrangement either side of the serine residues which accept xylose during glycosaminoglycan chain attachment is very important, because neither free serine nor serine in proteins other than proteoglycans act as xylose acceptors (BAKER, RODÉN and YAMAGATA, 1971).

MARSHALL (1972), studying the peptides containing serine residues associated with xylose, observed that glutamic acid was always present. The presence of glutamic acid (and also glycine) near the serine residues which were glycosylated has been reported also by WUSTEMAN and DAVIDSON (1975). They proposed that both glutamic acid and glycine formed part of a specialized sequence near serine residues which were destined to accept chondroitin sulphate chains. WUSTEMAN and DAVIDSON (1975) also reported that proteoglycans extracted from pig costal cartilage and subsequently digested by trypsin and chymotrypsin yielded peptides containing four chains of chondroitin sulphate. Analysis of the amino acid composition of these peptides indicated a relatively uniform spacing between all four glycosaminoglycan chains, with an average of eight amino acids between the serine residues to which chondroitin sulphate was linked. In addition, there was one unsubstituted serine residue for every two linked glycosidically.

Therefore, it seems likely that some form of repeating sequence of amino acids exists in proteoglycan core protein. Indeed, this supposition is not inconsistent with the current concept of an extended (not globular) structure of proteoglycan core protein, since repeat segments of amino acids are common in other extended proteins (STONE, et al., 1979).

Recently, the studies of WALTON, VOLGER and JAYNES (1979) on a protein core of bovine nasal cartilage obtained by cyanogen bromide cleavage and removal of glycosaminoglycans by hydrogen flouride treatment have confirmed earlier reports that proteoglycan core protein consists primarily of proline, serine, glycine and glutamic acid. Furthermore, the close proximity of glycine and glutamic acid residues to the serine residues which bind chondroitin sulphate was also confirmed. End group analysis of the peptides isolated for this study revealed valine to be the N-terminal amino acid. On the basis of a crude amino acid analysis obtained by selective proteolytic degradation of the bovine nasal cartilage proteoglycan protein core, WALTON, VOLGER and JAYNES (1979) speculated that the most likely conformation for this core protein was a flexible extended chain containing β-twists.

The flexible nature of proteoglycan core protein has been further supported by TORCHIA, HASSON and HASCALL (1981). Using ^{13}C nuclear magnetic resonance techniques, they demonstrated that both proteoglycan protein core and glycosaminoglycan chains have approximately the same degree of flexibility. Their studies also indicated whole proteoglycan molecules to be flexible with restrictions on flexibility occurring at the carbohydrate-protein link regions (i.e. serine residues).

OLIGOSACCHARIDES

Apart from glycosaminoglycan side chains, smaller oligosaccharide chains have also been demonstrated in proteoglycans from a variety of sources. THONAR and SWEET (1979) were the first to report the existance of such oligosaccharides and described them as integral components of bovine articular cartilage proteoglycans. Since then oligosaccharides have also been observed in proteoglycans from cultures of chick limb bud chondrocytes (DE LUCA, et al., 1980) and Swarm rat chondrosarcoma cultures (LOHMANDER, et al., 1980).

From these initial studies, two general classes of oligosaccharides have been described. One, an O-glycosidic-linked class, whose structure appears to represent the moiety through which keratan sulphate is linked to cartilage proteoglycan core protein. The other, a mannose-rich class which is linked to proteoglycan protein core via N-glycosidic bonds.

The mannose-rich oligosaccharides seem to have a composition similar to that of a number of other glycoproteins. In particular, they are similar to the mucous glycoproteins which contain oligosaccharides linked to protein by N-glycosidic bonds to asparagine (KORNFELD and KORNFELD, 1976). Indeed, the mannose rich oligosaccharides are resistant to reduction by alkaline borohydride and are, therefore, most likely bound to asparagine residues in the proteoglycan core protein (DE LUCA, et al., 1980). On the other hand, the oligosaccharides which are bound to the core protein of proteoglycans via an O-glycosidic bond are labile to alkaline hydrolysis and hence are probably bound to either serine or threonine residues (DE LUCA, et al., 1980).

Following extensive alkaline and proteolytic degradation of the core proteins of proteoglycans obtained from cultures of a Swarm rat chondrosarcoma, LOHMANDER, et al. (1980), proposed that the mannose rich oligosaccharides (N-linked) were mainly located in the hyaluronic

acid binding region, whereas, the majority of the O-linked oligosaccharides were present in the chondroitin sulphate peptide fragments.

Whilst the structures for both the O-linked and N-linked oligosaccharides of cartilage proteoglycans have been described (DE LUCA, et al., 1980; LOHMANDER, et al., 1981; NILSSON, et al., 1982), their function has not yet been determined. Nonetheless, age related changes in the distribution of oligosaccharides in bovine articular cartilage proteoglycans have been reported (SWEET, THONAR and MARSH, 1979; GARG and SWANN, 1981; SANTER, WHITE and ROUGHLEY, 1982). In these studies, an inverse relationship between O-linked oligosaccharides and keratan sulphate content of the proteoglycans at different ages was noted.

Oligosaccharides have also been identified in non-cartilagenous tissues. For example, the presence of glycoprotein type oligosaccharides in proteoglycans extracted from bovine aortae has recently been reported (KAPOOR, et al., 1981; SCHMIDT, et al., 1982). Oligosaccharides have also been isolated in a chondroitin sulphate proteoglycan from brain (FINNE, et al., 1979), rat ovarian proteoglycans (YANAGASHITA, et al., 1979) as well as in proteoglycans extracted from chromaffin granule matrix of bovine adrenal medulla (KIANG, et al., 1982).

PROTEOGLYCAN MOLECULAR SIZE

The molecular weights of proteoglycans are very variable, with reported values ranging from 10^5 to several million for proteoglycans extracted from various tissues (HASCALL and SAJDERA, 1970; PASTERNACK, VEIS and BREEN, 1973; OLDBERG, KJELLEN and HÖÖK, 1979). However, such variability is not seen only between different tissues. Indeed, proteoglycans from the same tissue source may be heterogenous. For example, hyaline cartilage

proteoglycans are polydisperse in both length (ROSENBERG, HELLMANN and KLEINSCHMIDT, 1975; THYBERG, LOHMANDER and HEINEGÅRD, 1975) and molecular weight (HASCALL and SAJDERA, 1970), with, one report citing a range in molecular weight for these proteoglycans of 1×10^6 to 4×10^6 (REIHANIAN, et al., 1979).

A similar polydispersity with respect to molecular weights has also been reported for a population of proteoglycans extracted from articular cartilage and was related to the amount of chondroitin sulphate present (ROSENBERG, et al., 1976). That is, as the fractions within this group of proteoglycans increased in molecular weight, there was a proportional increase in the number of chondroitin sulphate chains. These increases were also reflected by a parallel increase in serine and glycine content within the protein core. This latter observation supports the hypothesis that the increase in size was related to increased numbers of chondroitin sulphate chains rather than size increases within these chains.

The degree of proteoglycan molecular weight heterogeneity does not, however, depend only upon quantitative differences in the glycosaminoglycan and protein components. Rather, qualitative differences may also prevail. For example, proteoglycans may possess long protein cores with short carbohydrate side chains, or, conversely, short protein cores with long carbohydrate chains. This concept has been formulated from electronmicrographic studies of various proteoglycan molecules. The short protein core form possessed a central filament of 170-190 nm bearing 20-22 side chains each 52-54 nm long, whereas the long protein core form possessed a central filament of 320-340 nm long bearing 34 side chains of a similar dimension to the shorter proteoglycan (WELLAUER, WYLER and BUDDECKE, 1972).

PROTEOGLYCAN INTERACTIONS

Carbohydrate-Carbohydrate Interactions

In a previous section (page 21) the various problems of analysing aggregating dermatan sulphate chains were discussed. Such complications, however, highlight the capacity of such macromolecules to interact *in vivo*. Indeed, the ability of polysaccharides to form complexes with each other should be of considerable biological interest with respect to their influence on other extracellular macromolecular interactions as well as their contribution to the overall gel or network nature of the extracellular matrices.

Using dermatan sulphate-substituted agarose gels, FRANSSON (1976) demonstrated that copolymeric dermatan sulphate (i.e. those chains composed of L-iduronic acid-galactosamine disaccharide units as well as the "chondroitin sulphate-like" repeating unit of D-glucuronic acid-galactosamine) could bind to these gels. Since then, the binding of copolymeric, as well as homopolymeric glycosaminoglycans (i.e. those comprised of only one type of repeating disaccharide unit), to dermatan sulphate substituted gels has been demonstrated. Chondroitin sulphate 4, heparan sulphate and heparin all demonstrate interaction with gels substituted with copolymeric dermatan sulphate glycosaminoglycans, whereas, chondroitin sulphate 6, hyaluronic acid and keratan sulphate do not (FRANSSON, *et al.*, 1979). Such binding of copolymeric or homopolymeric glycosaminoglycans to copolymeric glycosaminoglycan substituted gels is most pronounced if the copolymer on the gel contains similar proportions of L-iduronic acid and D-glucuronic acid residues.

More recently, gel chromatography and light scattering methods have demonstrated that certain heparan sulphate chains are able to self associate (FRANSSON, NIEDUSZYNSKI and SHEEHAN, 1980). This property was subsequently shown to correlate closely with the presence

of alternating or mixed sequences of glucuronic acid-N-acetylated glucosamine; glucuronic acid-N-sulphated glucosamine and iduronic acid-N-sulphated glucosamine disaccharide units (FRANSSON, HAVSMARK and SHEEHAN, 1981). These characteristics are not dissimilar to those of self aggregating dermatan sulphate chains. Indeed, it appears that the presence of both glucuronic acid and iduronic acid residues within the chain is a common feature, and may well dictate whether a chain is capable of self aggregation.

Presently, the biological significance of these interactions is not understood. Nonetheless, it could be postulated that since dermatan sulphate-dermatan sulphate interactions similar to those observed using dermatan sulphate substituted gels, have been noted in extracts from fibroblast cultures (MALMSTRÖM, et al., 1975) then similar interactions may occur in vivo within connective tissues. Indeed, in fibrous connective tissues, dermatan sulphate proteoglycans are characteristic components of their extracellular matrices (FRANSSON, 1970) and any self aggregation of these molecules could play a functional biosynthetic or biophysical role similar to that of the hyaluronic acid-proteoglycan interaction observed in cartilage.

On the other hand, heparan sulphate, which is a recognized cell surface macromolecule also demonstrates self aggregating capacities, and could be implicated functionally in cell-cell contact (FRANSSON, et al., 1980). This concept has recently been supported by the observations of FRANSSON, SJÖBERG and CHIARUGI (1981), that heparan sulphates from transformed human fibroblasts do not demonstrate self associating properties. This may explain, in part, the altered cell-cell interactions observed for these transformed fibroblasts.

Hyaluronic Acid-Proteoglycan Interactions

In cartilage, most proteoglycans are aggregated with hyaluronic acid (HARDINGHAM and MUIR, 1973) and form an important part of the extracellular matrix of this tissue (ANDERSON and SAJDERA, 1971). These aggregates are formed by specific non-covalent interactions of proteoglycans (i.e. core protein with glycosaminoglycan side chains covalently bound; often termed a proteoglycan subunit) to single chains of hyaluronic acid (HARDINGHAM and MUIR, 1972; 1974; HASCALL and HEINEGÅRD, 1974). The resultant complex is thus termed a proteoglycan aggregate. On the basis of calculations, it has been postulated that up to 200 proteoglycan subunits, each with an average molecular weight of 2×10^6, can bind to a single hyaluronic acid chain of molecular weight 1.6×10^6 to produce aggregates up to 4 μm long and of molecular weight 350×10^6 (HARDINGHAM, et al., 1981).

The binding site of the proteoglycan subunit core protein to hyaluronic acid is visualized as a compact region of peptide located at one end of the protein chain (PERKINS, et al., 1981). This appears to be of invariant amino acid composition and molecular weight to which no glycosaminoglycans are bound (HEINEGÅRD and HASCALL, 1974 a & b). HARDINGHAM, et al. (1976) confirmed that the binding of proteoglycan subunit to hyaluronic acid was a function of the protein core, and was dependent upon disulphide bridges, intact arginine and tryptophan residues, as well as the ε-amino groups of lysine. However, subsequent reports have indicated that not only is a specific amino acid sequence within the proteoglycan core protein required for hyaluronic acid-proteoglycan interaction, but the hyaluronic acid carboxyl anions must be in a specific spatial orientation in order for such an interaction to occur (CHRISTNER, BROWN and DZIEWIATKOWSKI, 1977, 1978, 1979; NIEDUSZYNSKI, et al., 1980).

In these studies, only hyaluronic acids of decasaccharide size or larger were demonstrated to permit proteoglycan subunit interaction. The decasaccharide sequence required a glucuronic acid residue at the non-reducing end and an N-acetyl glucosamine residue at the reducing end ([-glucuronic acid-N-acetyl glucosamine-] $_5$). If these decasaccharides were enzymatically modified in specific ways it appeared that at least one other N-acetyl glucosamine residue together with five unmodified carboxyl groups were required for aggregate formation. Additional residues beyond the decasaccharide sequence did not interact with proteoglycan or influence its association with hyaluronic acid.

Apart from the specific regions of the proteoglycan core protein and hyaluronic acid, some lower molecular weight glycoproteins are also integral parts of these aggregates. They are termed "link proteins" (HASCALL and SAJDERA, 1970; HARDINGHAM, 1979; TANG, et al., 1979). These appear to be closely associated with the binding of cartilage proteoglycans to hyaluronic acid (stabilizing the bond) effectively locking the proteoglycan onto the hyaluronic acid chain. The importance of link protein in stabilizing the hyaluronic acid-proteoglycan aggregate was first reported by GREGORY (1973). He noted that the complex was not stable in the ultracentrifuge under physiological conditions unless these proteins were present. Indeed, in the absence of link protein, proteoglycan subunits bind reversibly to hyaluronic acid. Such interactions are open to competition with hyaluronic acid oligosaccharides. However, if link protein is present, there is no competitive binding with hyaluronic acid oligosaccharides and the aggregate is no longer in equilibrium with its dissociation products. That is, the aggregate becomes stabilized in the presence of link proteins (HARDINGHAM and MUIR, 1974; HARDINGHAM, 1979).

In cartilagenous tissues, link protein exists in two molecular forms which are structurally related. Both contribute actively to the overall stabilizing effect and are present as one molecule of link per molecule of proteoglycan (BAKER and CATERSON, 1979; KIMURA HARDINGHAM and HASCALL, 1980).

Functionally, the proteoglycan-hyaluronic acid aggregates of cartilage are assumed to enable this tissue to withstand compressive forces. Compression causes displacement of water from the extracellular matrix, however, because of the high osmotic pressure of the proteoglycan-hyaluronic acid aggregates, removal of the compressive force causes the opposite to occur (HASCALL, 1977). As a result, these aggregates give cartilage its resilience, ability to deform with compression under load, as well as return to its original shape on removal of the force.

Until recently, the capacity of proteoglycans to form very large complexes with hyaluronic acid was considered a unique property of hyaline cartilage. Nonetheless, aggregatable proteoglycans have also been observed in a number of other tissues which are either related to, or closely associated with cartilage. For example, proteoglycans from intervertebral disc, nucleus pulposus, annulus fibrosus and cultured dedifferentiated chondrocytes have all demonstrated some ability to aggregate with hyaluronic acid (PEARCE and GRIMMER, 1976; ADAMS and MUIR, 1976; STEVENS, et al., 1979; OEGEMA and THOMPSON, 1981).

Recently, however, proteoglycans from several non-cartilagenous tissues have also been demonstrated to have some capacity to interact with hyaluronic acid. For example, a chondroitin sulphate proteoglycan from glial cells (NORLING, et al., 1978), a chondroitin sulphate-dermatan sulphate proteoglycan from aorta (McMURTREY, et al., 1979; OEGEMA, HASCALL and EISENSTEIN, 1979) and an uncharacterized proteoglycan

from human gingival epithelium (WIEBKIN, BARTOLD and THONARD, 1979) have all demonstrated some aggregatability with hyaluronic acid.

Although most of these non cartilagenous proteoglycan-hyaluronic acid interactions have been demonstrated in vitro, the possibility that they occur in vivo cannot be discounted. Indeed, it appears that several proteoglycans (apart from those found in cartilage) have the capacity to interact with hyaluronic acid, and, therefore, proteoglycan-hyaluronic acid aggregates may be more widely distributed throughout the tissues than has been previously thought. If this is the case, then they may be of wider physiological importance than that presumed for cartilage alone.

Collagen-Proteoglycan Interactions

One of the most widely studied biological macromolecular interactions is that between the two major extracellular connective tissue components, collagen and proteoglycans. Nonetheless, despite intense investigation, the precise nature and physiological significance of these interactions is still unclear. (For reviews see: JACKSON and BENTLEY, 1968; MATHEWS, 1970; ÖBRINK, 1975; PODRAZKÝ, 1981).

MEYER, PALMER and SMYTH (1937) and MEYER and SMYTH (1937) were the first to report an interaction between collagen and glycosaminoglycans following the observation that chondroitin sulphate formed insoluble complexes when interacted with gelatin in vitro. Much later, MATHEWS (1965 b), studied the interactions of solubilized collagen with hyaluronate and chondroitin sulphate and noted that the resultant complexes were reversible. By this stage it was generally accepted that electrostatic interactions were responsible for such complex formation, being principally mediated by sulphate and carboxyl groups of the glycosaminoglycans and the basic amino groups of collagen (MATHEWS and DECKER, 1968 b). This concept has since been confirmed by a

number of workers using a variety of methods (ÖBRINK, 1973 a; TOOLE, 1976; PODRAZKÝ, et al., 1971; LEE-OWN and ANDERSON, 1976). Ionic binding also appears to be responsible for similar complexes formed between collagen and proteoglycan (STEVENS, JACKSON and BROADY, 1968, 1969).

Therefore, since such complex formation is considered to be mainly due to cooperative electrostatic forces, the net charge and the charge distribution along both the proteoglycan (or glycosaminoglycan) and collagen molecules would be expected to play important roles in the strength of these interactions. Indeed, on the basis of this, NIMNI (1975) proposed a model for collagen-proteoglycan interactions, which described induced conformational changes in proteoglycans caused by specific surface characteristics of collagen molecules. He suggested that hydroxylysine with various degrees of glycosylation as well as ionic interactions between the peptide-bound ε-amino group of lysine and hydroxylysine and the negatively charged groups of the proteoglycans (namely the glycosaminoglycan side chains) would be of particular significance. Furthermore, stabilization by hydrogen bond formation between glycosaminoglycans and glycosidically attached glucosyl-galactosyl residues together with salt linkages were implicated in this model (see Figure 1.2). Such a model attempted to explain how proteoglycans might "coat" collagen.

However, it seems that NIMNI's model is oversimplistic because the number of interactive sites on a collagen molecule is restricted (ÖBRINK and SUNDELÖF, 1973) and hence proteoglycans do not completely "coat" collagen as suggested above. Indeed, the association of negatively charged glycosaminoglycans is restricted only to those portions of the collagen molecule which carry a net positive charge. Furthermore, despite the apparent high negative charge distribution along glycosaminoglycans, these too, have a limited number of sites which are interactive with collagen (ÖBRINK and SUNDELÖF, 1973).

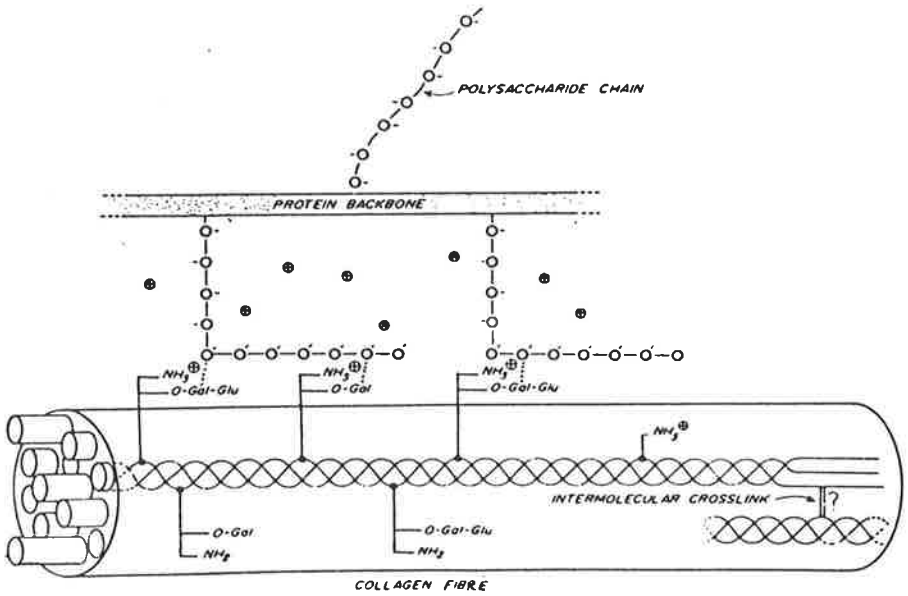


Figure 1.2 Model for proteoglycan-collagen interaction. Based on the model proposed by NIMNI (1975).

Interactions of collagen and proteoglycan in cartilage. This shows the potential role of the lysine and hydroxylysine residues as well as the glycosidically bound carbohydrate in such interactions. Ionic interactions as well as hydrogen bond formation could contribute significantly to the overall interaction.

Therefore, the concept of strong electrostatic interactions between collagen and proteoglycan has recently been questioned (VEIS and HELSETH, 1982). By calculating the net charge distribution along type I collagen under physiological conditions, they concluded that this molecule does not exhibit high polyelectrolyte characteristics. Indeed, on the basis of these calculations, it would seem that there is only a net positive charge at the region near the amino terminal. Furthermore, they propose, that despite the obvious high negative charge of glycosaminoglycans, most of this charge would be shielded by "*localized*" micro ions at physiological pH and ionic strength. Consequently, they claim that any interaction between collagen and proteoglycan would be electrostatically very weak and therefore of questionable importance with respect to its reported importance in fibril formation, organization and stabilization.

Nonetheless, the fact remains that collagen and proteoglycans do undergo complex formation both in vivo and in vitro. In general, all the sulphated glycosaminoglycans, with the exception of keratan sulphate, bind to collagen within the range of physiological pH and ionic strength. No binding has been observed for hyaluronate (GOH and LOWTHER, 1966; LOWTHER and BAXTER, 1966; ÖBRINK, 1973 a; GELMAN and BLACKWELL, 1974). Furthermore, of the glycosaminoglycans which do form complexes with collagen, those which are rich in L-iduronic acid residues (in particular, dermatan sulphate) appear to form stronger complexes with collagen (TOOLE and LOWTHER, 1966; LOWTHER, TOOLE and MEYER, 1967). The close association of dermatan sulphate with collagen in vivo was confirmed later by the resistance of this glycosaminoglycan to low salt extraction of connective tissue; usually remaining with the insoluble tissue residue (TOOLE and LOWTHER, 1968; ÖBRINK, 1973 a).

Proteoglycan-collagen complex arrangements *in vivo*, may also vary with respect to the type of collagen (JUNQUIERA, *et al.*, 1980). For example, adult dermis, which contains predominantly type I collagen is characteristically rich in dermatan sulphate, whilst cartilage, which contains predominantly type II collagen, is richest in chondroitin sulphate (TOLEDO and DIETRICH, 1977). Similarly, the presence of dermatan sulphate, chondroitin sulphate and heparan sulphate in tissues containing predominantly collagen types I, II and III respectively have been reported recently (JUNQUIERA, TOLEDO and MONTES, 1981). However, whilst these observations implicate a correlation between collagen type and glycosaminoglycan species, they are only circumstantial on the basis of histological and limited biochemical assessment and consequently do not demonstrate unequivocally physical or chemical complex formation.

Functionally, proteoglycan-collagen complex formation has been implicated in collagen fibre formation *in vitro*. The exact nature of such an effect is not clear since the results of many studies have been varied and at times contradictory (reviewed by ÖBRINK, 1975). For example, proteoglycans, at various ionic strengths and pH may delay (TOOLE, 1969; ÖBRINK, 1973 b), accelerate (LOWTHER, TOOLE and HERRINGTON, 1970) or have no effect (GROSS and KIRK, 1958; LOWTHER and NATARAJAN, 1972) on collagen fibre formation. Such inconsistencies may be explained, in part, by differences in ionic strength, pH, collagen preparations used and phase of fibre formation at which these experiments were carried out. With respect to the latter, ÖBRINK (1973 b) studied, *in vitro*, the effect of adding various glycosaminoglycans and proteoglycans before and after the nucleation phase of collagen fibrillogenesis at 37°C under physiological conditions. Under such conditions, chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin and proteoglycans comprised of chondroitin sulphate and dermatan sulphate, when added before

the nucleation phase, accelerated collagen fibre formation. On the other hand, when added after the nucleation phase, chondroitin sulphate, heparan sulphate and proteoglycans of chondroitin sulphate and dermatan sulphate delayed fibre formation. It was thus concluded, that aggregation, or nucleation, of in vitro assembled collagen was of prime importance with respect to the influence of complexing glycosaminoglycans of proteoglycans. This, it was claimed, explained in part, some of the earlier contradictory findings. Similarly, OEGEMA, et al. (1975) have noted that the organization of collagen fibrils in tissues is related to both the kinds of proteoglycans (i.e. proteoglycan subunit or proteoglycan aggregate) as well as the amounts of proteoglycans present. They also demonstrated that the effect of proteoglycans on collagen fibril formation varied depending on which stage of nucleation they were added.

The thermal stability of collagen can also be influenced by proteoglycans and glycosaminoglycans. For example, in vitro studies have demonstrated that both sulphated and non-sulphated glycosaminoglycans interact with collagen, under acidic conditions, causing a rise in the melting temperature of collagen from 38°C to 46°C (GELMAN and BLACKWELL, 1973; 1974; BLACKWELL and GELMAN, 1975). However, the relevance of these studies to those interactions which occur in vivo between collagen and proteoglycan is questionable since the above studies were performed at acid pH and considered glycosaminoglycans alone. Indeed, more recently, SNOWDEN and SWANN (1980) demonstrated quite the opposite to the above studies with both proteoglycans and glycosaminoglycans causing a reduction in the thermal stability of in vitro assembled collagen fibrils at physiological ionic strength and pH. Nonetheless, some indirect evidence does exist which suggests that both proteoglycans and glycosaminoglycans may stabilize in vivo

assembled collagen in some tissues. SNOWDEN (1982) has demonstrated that removal of proteoglycan and glycosaminoglycans from bovine articular cartilage by pretreatment with enzymes (hyaluronidase) resulted in decreased thermal stability of the collagen. The stability of such pretreated collagen was slightly increased when measurements were made in the presence of chondroitin sulphate, indicating a stabilizing effect of chondroitin sulphate.

Other studies on proteoglycan-collagen interactions have considered their ultrastructural arrangements by electronmicroscopy (MATUKAS, PANNER and ORBISON, 1967; ANDERSON and SAJDERA, 1971; CAMPO and PHILLIPS, 1973; RUGGERI, DELL'ORBO and QUACCI, 1975). More recently SCOTT (1980) used a cationic phthalocyanin-like dye on gluteraldehyde or formaldehyde fixed foetal and adult rat tail tendon to visualize the attached proteoglycans. Such a technique was claimed to overcome earlier proteoglycan localization problems by being more specific for proteoglycans, and causing a partial collapse of these molecules by dehydration, thus permitting better visualization. Furthermore, this dye could be used in a critical electrolyte system to allow selective staining of glycosaminoglycans. Tissues treated in this fashion exhibited proteoglycan arranged in orthogonal arrays on collagen fibres. No proteoglycan was observed within the collagen fibres The orthogonal arrangement consisted of both vertical and horizontal components with the horizontal component being regularly separated by the collagen band repeat distance. It was thus concluded that collagen and proteoglycan demonstrate a specific interaction. In a subsequent study, on rat tail tendon collagen, SCOTT and ORFORD (1981), using the same dye as before, critical electrolyte concentration techniques and selective enzymatic (hyaluronidase) digestion, confirmed

the above observations, as well as localizing the tranverse (horizontal) elements of the proteoglycans almost exclusively at the d band in the gap zone. This latter finding is of particular interest in the light of the model of proteoglycan-collagen interaction offered by VEIS and HELSETH (1982). They proposed that one of the few regions on the collagen fibre where a sufficient net positive charge existed was the edge of the hole (gap) region and was therefore a likely area for proteoglycan interaction. Regular proteoglycan association along collagen fibrils has been also reported using immunoelectron microscopy (POOLE, et al., 1982).

A similar, but less well defined arrangement of proteoglycan with collagen has been proposed by VIDAL (1980). Using birefringence techniques, proteoglycans were visualized to be associated with collagen with their long axis (protein core) being sloped with respect to the long axis of the collagen fibres while the glycosaminoglycan chains exhibited their long axis parallel to that of the collagen fibres.

Therefore, whilst there appears to be no doubt that collagen and proteoglycans do form complexes, the precise manner in which they occur is only beginning to be elucidated. Indeed, the interaction is complicated and it would seem very likely that factors other than cooperative electrostatic interactions would be important. For example, physical parameters such as thermodynamic interactions, steric exclusion and molecular entanglement have not been considered in this section but probably play some important roles in the complex formation between proteoglycans and collagen.

PROTEOGLYCAN DEGRADATION

Degradation of the extracellular matrix of connective tissues may occur under a variety of physiological and pathological conditions. Such degradative processes result in alterations to the two major extracellular components of the matrix, namely collagen and proteoglycan. As one model for understanding the changes imposed upon proteoglycans under these conditions, cartilage proteoglycans have been extensively studied.

The progression of proteoglycan degradation has been studied by WASTESON, LINDAHL and HALLÉN (1972). They noted that costal cartilage proteoglycans which had been partially degraded in vitro, contained glycosaminoglycans which were unaffected with respect to molecular weight and size. In all cases protein cleavage had occured. They therefore proposed that proteolysis preceded carbohydrate digestion during cartilage proteoglycan degradation.

Since this study, most attention regarding cartilage proteoglycan degradation has focused upon proteolytic enzymes with only minor consideration being given to carbohydrases. Indeed, the proteolytic digestion of bovine nasal cartilage proteoglycans by a variety of enzymes has been extensively studied by ROUGHLEY and BARRETT (1977) and ROUGHLEY (1977, 1978). The enzymes studied were :- papain, trypsin, cathepsin G, cathepsin D, chymotrypsin, pancreatic elastase, lysosomal elastase, cathepsin B, thermolysin, pronase and papain. Analysis of the fragments produced by pretreatment of the cartilage proteoglycans with these enzymes indicated that all these enzymes could degrade proteoglycans. However, they varied in their capacity to fragment the proteoglycan molecule. That is, some cleaved the protein core in only a limited number of sites leaving peptide fragments with many glycosaminoglycan chains, whilst others (which were considered to be more

effective in degradation) cleaved the protein core at many sites resulting in smaller peptides with fewer glycosaminoglycan chains.

Furthermore, it is important to note that enzymes operate optimally at a variety of pH's and therefore may vary in the degree of their activity in vivo. Indeed, of the above enzymes studied, cathepsin B and cathepsin D have been found to be most active at an acid pH (DINGLE, 1973; BAYLIS and ALI, 1978), whereas, cathepsin G and neutrophil elastase are the most active at neutral pH (BARRETT, 1978). A metal dependant neutral proteinase which demonstrates elastase-esterase, trypsin-like and chymotrypsin-like activities has also been implicated in cartilage proteoglycan degradation (MALEMUD, et al., 1979).

Nonetheless, whilst these enzymes do vary in pH dependence for optimal activity of cartilage proteoglycan degradation, current concepts indicate that both acidic and neutral proteinases may be potentially involved in vivo. For example, the role of neutral proteinases in cartilage proteoglycan degradation has been supported by the demonstration of proteoglycan activity within articular cartilage at physiological pH (SAPOLSKY, HOWELL and WOESSNER, 1974). However, whilst the average pH of cartilage extracellular matrix may be neutral under physiological conditions, normal metabolic activity of the cells may produce transient acidic enviroments in their immediate vincinity. Indeed, if this is the case, then, since articular cartilage is rich in the acid dependent cathepsin D (SAPOLSKY, et al., 1973), the action of this enzyme may be enhanced in an acidic pericellular environment.

Mediators and inhibitors of enzyme activity have also been implicated in the regulation of proteoglycan degradation. For example SAKLATVALA and DINGLE (1980) have reported the presence of an acidic protein, of molecular weight 20,000, termed "catabolin", in human rheumatoid synovium, which stimulates chondrocytes to produce proteoglycan degrading enzymes. This protein is also produced by fibroblasts and activated monocytes in vitro. A similar protein which stimulates chondrocytes to produce proteoglycan degrading enzymes and is released by macrophages has also been described (RIDGE, ORONSKY and KERWAR, 1981). Both of these proteins are found in elevated amounts in cartilage affected by rheumatoid arthritis, trauma and other mechanisms of tissue damage (DINGLE, 1981). Nonetheless, whilst chondrocytes do release proteinases which are capable of degrading proteoglycans, they also simultaneously release inhibitors specific for these enzymes (ROUGHLEY, MURPHY and BARRETT, 1978; KNIGHT, et al., 1979). The proteinases are, therefore, present in a latent state and may be activated only by chemical or enzymatic removal of the inhibitor. Consequently, whilst the chondrocytes may produce all the enzymes required to degrade cartilage proteoglycans, it is not until the inhibitor is removed that they become active. Furthermore, if the inhibitor is either not released in sufficient quantity or if it is rapidly degraded then a greater proportion of the proteinases, normally released by the chondrocytes, will be in an active form. This could lead to an increase in proteoglycan degradation as seen in some pathological conditions.

On the basis of these observations, VAES (1980) postulated that chondrocytes produce a basal level of neutral proteinase, which is used during the normal physiological turnover of cartilage proteoglycan, whilst the enhanced degradation of proteoglycans seen in pathological conditions is related to either an increase in the presence of agents such as catabolin or total inactivity of the proteinase inhibitors.

The relevance of the above studies to soft tissue degradation is unclear since there are vast qualitative and quantitative differences between the extracellular matrices of cartilage and soft tissues. In particular, the proteoglycans which comprise these tissues are dissimilar to those of cartilage with respect to their composition as well as their molecular interactions. Nonetheless, the identification of proteoglycan degrading enzymes within cartilage in healthy and pathological conditions would indicate that similar enzymes could be present within soft tissues, although their nature and mode of action may not necessarily be identical to the cartilage enzymes.

To date, there has been little information published relating to proteoglycan proteinases of soft tissues and in particular gingivae. Indeed, this is surprising since gingiva, which exists both in a healthy state as well as in a degenerating, chronically inflamed state (periodontal disease), would be expected to provide an excellent model for studying soft tissue degradation.

Recently, a neutral metallo-proteinase has been identified within inflamed gingival tissue (UITTO, APPELGREN and ROBINSON, 1981). However, whilst the substrate for this enzyme was presumed to be proteoglycan there was no confirmation of proteoglycan degrading

activity. However, EMBERY, OLIVER and STANBURY (1979) have demonstrated specific proteoglycan degradation in human gingiva *in vivo*. Under their conditions the protein moiety of the proteoglycan was partially degraded resulting in smaller molecular size but leaving the glycosaminoglycan chains relatively unaffected. They were, unable to isolate the responsible enzyme. The susceptibility of gingival proteoglycans has been further demonstrated by PURVIS, EMBERY and OLIVER (1981) and PURVIS and EMBERY (1981) *in vitro* using a neutral proteinase isolated from human polymorphonuclear leucocytes. Moreover, confirmation that gingival cells produce a proteoglycan degrading enzyme has been presented by HEATH, *et al.* (1982). This enzyme was, however, under their conditions found to be present in a latent form only.

The fate of the proteoglycans initially degraded by proteinases is still under investigation. Studies on the catabolism of chondroitin sulphate in animals imply that following proteoglycan protein core degradation, the glycosaminoglycan chains are relatively unaffected, remaining approximately the same size as for undegraded proteoglycans (WASTESON, LINDAHL and HALLÉN, 1972). Furthermore, the glycosaminoglycan chains have been observed to be removed from the tissues in an undegraded state and are later degraded in the liver or excreted from the body without undergoing any further degradation (REVELL and MUIR, 1972; WOOD, *et al.*, 1973). Evidence for phagocytosis of glycosaminoglycans at the site of degradation has also been reported (SAITO and UZMAN, 1971 a & b). More recently, the endothelial cells of the liver have been implicated in the degradation of hyaluronic acid injected intravenously into rabbits and humans (FRASER, *et al.*, 1981; FRASER, *et al.*, 1982; ERIKSSON, *et al.*, 1982).

The roles of tissue hydrolytic enzymes in degrading proteoglycans are confusing. Indeed, many hydrolytic enzymes have been identified in inflamed tissues (especially gingivae) and various claims made regarding their role in tissue degradation. In particular, of those identified in inflamed tissue, β-glucuronidase, aryl sulphatase and hyaluronidase have been implicated in the degradation of gingival proteoglycans (SCHULTZ-HAUDT, 1957; CABRINI and CARRANZA, 1960; GOGGINS, FULLMER and STEFFEK, 1968; INNES, 1974; PODHRADSKÝ, JÁNY and VELGOS, 1982). Nonetheless, many of these studies were performed when there was less understanding of proteoglycan chemistry than we have today, and virtually no precise data concerning the proteoglycan composition of gingiva. In the light of the current concepts described earlier, it is difficult to see how any of these hydrolytic enzymes are involved in the initiation of proteoglycan degradation. They may, however, be involved in subsequent breakdown of glycosaminoglycans after the early proteoglycan core-protein degradation by proteinase, although, the observations of EMBERY, OLIVER and STANBURY (1979) that glycosaminoglycans in inflamed gingiva remain relatively intact despite an abundance of such hydrolytic enzymes mittigate against such an hypothesis. Indeed, this is further strengthened by subsequent observations of EMBERY, OLIVER and STANBURY (1981) that intact glycosaminoglycans are found in gingival sulcular fluid, rather than glycosaminoglycan degradation products such as uronic acid containing oligosaccharides, N-acetylglucosamine or uronic acid monosaccharides.

Therefore, the most likely sequence of degradation of soft tissue proteoglycans would appear to be initial cleavage of the proteoglycan core protein followed by removal of glycosaminoglycan chains from the tissue to a site where they may be either further degraded or excreted. Obviously, the role of hydrolytic enzymes within these tissues remains obscure.

CHAPTER 2

GENERAL METHODS

GINGIVAL TISSUE

Specimens of human gingival tissue were obtained from two sources. Firstly, tissue specimens were obtained from gingivectomy on patients undergoing periodontal therapy. They had been previously subjected to presurgical preparation and were considered clinically free from gingival inflammation. Immediately prior to surgery the periodontal status of the tissues was assessed using the Gingival Index (LÖE and SILNESS, 1963) and the Periodontal Index (RAMFJORD, 1967). Only those specimens which were judged to be within the range of "healthy appearance" were utilized for subsequent analysis.

The other source of gingival tissue was obtained by gingivectomy on human cadavers which were not more than 8 hours old. The state of health of these specimens was determined by measurement of gingival sulcus pocket depth prior to removal. Gingivae of cadavers with pockets of 3 mm or less were excised.

Following excision of gingival tissue, the specimens were immediately placed into 10 mls of Ca^{++} and Mg^{++} free phosphate buffered saline containing 1 mM EDTA, pH 7.2 (APPENDIX 1).

Histological assessment was carried out on representative portions of each specimen to determine the degree of inflammation present, following routine haematoxylin and eosin staining (APPENDIX 2). Only those specimens demonstrating very mild inflammatory infiltration adjacent to the sulcular epithelium as well as an intact sulcular epithelial lining were used. Specimens with more advanced histological signs of inflammation were discarded.

Gingival specimens from cadavers were used only for the experiments described in Chapter 3. The work described in other chapters was performed on "healthy" gingivectomy specimens from patients receiving periodontal therapy. In this way degradation of glycosaminoglycans and proteoglycans

by autolysis was minimized, an important consideration with respect to molecular weight and size observations of these macromolecules.

SEPARATION OF EPITHELIUM AND CONNECTIVE TISSUE

A distinct cleavage of epithelium from its underlying connective tissue along the extremely convoluted basement membrane of gingiva provided the two sources of tissues for the studies on proteoglycans and glycosaminoglycans reported in this thesis.

Numerous techniques have been developed for separating ectodermal from mesodermal tissues. However, specimens free from residual epithelium or connective tissue have often been difficult to obtain from tissues such as skin and gingivae due to the presence of a highly convoluted basement membrane.

Microdissection of these two regions of gingivae has been attempted (BARTOLD, WIEBKIN and THONARD, 1982), however, uncontaminated specimens of gingival epithelium and connective tissue were not obtained with these methods (Figure 2.1). Other techniques have used enzymes such as elastase (NEIDERS and WEISS, 1970) or collagenase (FELTON, et al., 1965; EINBINDER, WALZER and MANDEL, 1966) as well as the combination of potassium thiocyanate and proteolytic enzymes (ARAYA, IMAGAWA and SHIZUYA, 1962). However, such pretreatment of tissues with enzymes assumes disaggregation of the intercellular matrix and the properties of proteoglycans could be affected by degradation of their protein cores. Hence, enzymatic treatment of gingival tissues to obtain separated epithelium and connective tissue may render the proteoglycans

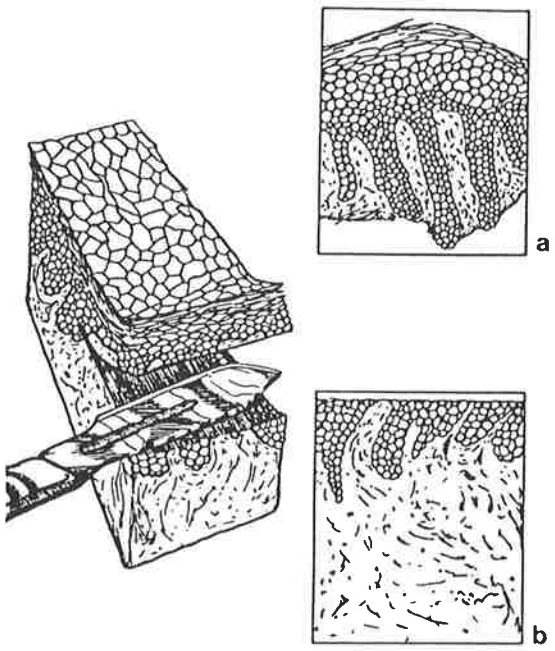


Figure 2.1

Diagram showing the separation of the apposing tissue types of gingivae by careful microdissection.

The schematic histological sections demonstrate the representative contamination of (a) connective tissue in an epithelial preparation and (b) epithelial rete peg remnants in connective tissue.

of the extracellular matrices unsuitable for the studies intended in this thesis. Furthermore, such pretreatment with enzymes tends to result in the release of isolated epithelial cells rather than intact epithelium (NEIDERS and WEISS, 1970).

Preparative procedures using chemicals alone have also been used to separate epithelium from connective tissue. One such method relied upon the removal of divalent cations by chelation (ZWILLING, 1955; BELL, SAUNDERS and ZWILLING, 1959). Distinct cleavage of oral epithelium from its underlying connective tissue has been demonstrated using this technique (SCALETTA and MAC CALLUM, 1972; McKENZIE and SWENDSON, 1977). Apart from obtaining uncontaminated samples of epithelium and connective tissue, additional advantages of this technique over enzyme pretreatment are: (1) potentially less disruption of the extracellular matrix components, and, (2) with this method, the basal lamina remains with the mesoderm, whereas with trypsin digestion the basal lamina remains with the ectoderm (GOEL and JURAND, 1968), thus minimizing the likelihood of residual connective tissue contamination in separated epithelial specimens.

A similar technique of chelation has been used in the present studies to separate gingival epithelium from its connective tissue. It was essentially a minor modification of the techniques described by SCALETTA and MAC CALLUM (1972) and McKENZIE, DABELSTEIN and ROED-PETTERSON (1979). Following excision and placement in the transport solution, the gingival tissue samples were cut into approximately 5 x 2 x 2 mm pieces and transferred to 5-10 ml of Ca^{++} and Mg^{++} free phosphate buffered saline containing 10 mM EDTA, pH 7.2 (APPENDIX 3). Incubation at 37°C for 30 minutes was sufficient to allow easy separation of gingival epithelium from connective tissue using

fine dissection forceps.

Cryostat or microtome sections were cut and routine staining with haematoxylin and eosin (APPENDIX 2) was carried out to verify separation and the degree of inflammatory infiltration, if any.

BIOCHEMICAL ASSAYS

Where the protocol concerned estimating relative levels of a molecular species within gingival tissue, for example, between epithelium and connective tissue, replicate experiments were performed; at least duplicate estimations were carried out.

Uronic Acid Estimation

The technique described by BLUMENKRANTZ and ASBOE-HANSEN (1973) was used.

Reagents:

1. Meta-hydroxydiphenyl solution. A 0.15% solution of meta-hydroxydiphenyl (Eastman Kodak Co., Rochester, New York) in 0.5% NaOH was prepared and stored in the refrigerator with an aluminium foil covering. This solution was remade every 6 months.
2. H_2SO_4/tetraborate solution. A 0.0125 M solution of sodium tetraborate in concentrated sulphuric acid.

Method:

To 0.2 ml of unknown and standards containing from 0.5 to 20.0 µg uronic acid, 1.2 ml of the sulphuric acid/tetraborate solution was added. The tubes were refrigerated in crushed ice. The mixture was then shaken in a Vortex mixer and the tubes heated in a boiling water bath for 5 minutes. After cooling in crushed

ice for 5-10 minutes, 20 µl of the m-hydroxydiphenyl reagent was added. The tubes were shaken and the colour allowed to develop for 5 minutes. Absorbance measurements were made in a Hitachi UV/Vis spectrophotometer (Model 139) at 520 nm.

Standards of D-glucuronic acid (sigma Chemical Co., St. Louis, U.S.A.) were used in most cases; however, for the experiments described in Chapter 4, standard samples of hyaluronic acid were used (APPENDIX 7).

Galactose Estimation

The secondary blue colour which develops after 24-48 hours in the cysteine-sulphuric acid method of DISCHE (1962) was used to assay galactose.

Reagents:

1. 0.05% mannose solution.
2. Sulphuric Acid: six parts concentrated H_2SO_4 and 1 part H_2O (v/v).
3. Cysteine Hydrochloride: 3% cysteine hydrochloride (Sigma Chemical Co., St. Louis, U.S.A.) prepared weekly and stored at 4^oC.

Method:

To 0.8 ml of unknown, standards (20-200 µg galactose) and blank 0.2 ml of 0.05% mannose was added.

To this, 4.5 ml of chilled sulphuric acid solution was added slowly with constant shaking in an ice bath to control temperature fluctuations. The tubes were then transferred to a water bath of room temperature for a few minutes, then to a (vigorously boiling) water bath, maintained throughout at 100^oC. After being heated for exactly 3 minutes, the tubes were placed in a water bath at room temperature. 0.1 ml of cysteine hydrochloride solution was then added and the contents

of each tube mixed thoroughly in a Vortex mixer. This mixture was allowed to stand at room temperature for 24-48 hours after which the yellow colour produced by hexoses in the 3 minute cysteine reaction changes to a blue colour in the presence of galactose. Absorbance measurements were made in a Hitachi UV/Vis Spectrophotometer (Model 139) at 600 nm.

Standards of D(+) galactose (Ajax Chemicals Ltd., Sydney, Australia) were prepared to obtain standard curves.

Protein Estimation

The technique of LOWRY, et al. (1951) was followed.

Reagents:

Solution 1: 2% Na_2CO_3 in 0.1 M NaOH.

Solution 2: 0.5% $CuSO_4 5H_2O$ in 1% sodium tartrate. This solution degrades quickly and must, therefore, be made up daily by mixing equal volumes of 1% $CuSO_4 5H_2O$ and 2% sodium tartrate.

Solution 3: An alkaline copper solution is made by mixing 50 parts of Solution 1 with 1 part of Solution 2 (v/v). This solution also is not stable and must be made up daily.

Folin's Reagent: Folin and Ciocalteu's phenol reagent, (B.D.H. Chemicals, Australia) was, first regenerated by adding 3 drops of bromine to 15 ml of reagent and boiling until no bromine remained. This solution was then diluted with 5 parts water to 4.7 reagent (v/v).

Method:

To 0.1-0.3 ml of sample and standards (50-500 µg protein/ml) 2.5 ml of solution 3 was added and mixed thoroughly. This was allowed to stand for 12-15 minutes at room temperature to permit reaction of copper with protein. 0.25 ml of the diluted Folin's reagent was added to the tubes and was immediately mixed vigorously. This mixture was allowed to stand for 30 minutes for colour development.

Absorbance measurements were made in a Hitachi UV/Vis Spectrophotometer (Model 139) at 700 nm.

Bovine Serum Albumin (Fraction V) was used as the standard in all cases.

Protein levels were also measured by direct absorbance at 280 nm. However, this procedure was never used for quantitative analyses, rather, it was used for qualitative analyses of column effluents.

CHAPTER 3

GLYCOSAMINOGLYCANS OF HUMAN GINGIVAL EPITHELIUM CONNECTIVE TISSUE

Isolation, Identification and Quantitation

INTRODUCTION

Since information regarding the proteoglycans of human gingivae is conflicting and there are no reports concerning the presence of these macromolecules in human gingival epithelium, the initial stage of this investigation into the nature of proteoglycans in adult human gingival epithelium and connective tissue was to identify and quantitate the glycosaminoglycan components of these two closely apposed tissues.

The analyses of gingival glycosaminoglycans were performed following enzymatic degradation of separated epithelium and connective tissue to release the polysaccharides. Subsequent identification of molecular species was achieved by electrophoresis as well as selective enzymatic degradation of specific glycosaminoglycans.

Currently, electrophoretic systems using cellulose acetate are the most widely used. Celite (GARDELL, GORDON and AQVIST, 1950), paper (FOSTER and PEARCE, 1961) and agarose (DIETRICH, McDUFFIE and SAMPIO, 1977) have also been used, but with poorer resolution. Nonetheless, despite the wide acceptance of cellulose acetate systems, they are still limited in their ability to adequately separate all seven known mammalian glycosaminoglycans in a simple, continuous, monodimensional system. In particular, the separation of chondroitin sulphates 4 and 6 has proven difficult due to their very similar structural and ionic properties. Consequently, there are innumerable reports regarding the separation of glycosaminoglycans on cellulose acetate using various buffers, pH's, discontinuous and multidimensional electrophoresis systems.

Following an extensive search of the literature concerning the electrophoretic separation of glycosaminoglycans, four systems were

chosen and tested as being the most likely to achieve separation of gingival glycosaminoglycans. The choice was based from collected published data which indicated that the possible glycosaminoglycan species in both human and animal gingivae were hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphates 4 and 6. The systems tested were:

a) 0.1 M pyridine-formic acid buffer, pH 3.0 (HERD, 1968).

b) 0.1 M barium acetate buffer, pH 8.0 (WESSLER, 1968).

c) 0.2 M calcium acetate buffer, pH 7.2 (STANBURY and EMBERY, 1977).

d) two dimensional system (HATA and NAGAI, 1972).

Of those tested, the system utilizing calcium acetate at pH 7.2 produced the best separation of glycosaminoglycan standards of hyaluronic acid, heparan sulphate, dermatan sulphate, chondroitin sulphate 4 and chondroitin sulphate 6.

Once this had been established, the system was slightly modified to allow rapid separation with maximum resolution. The gingival glycosaminoglycans were thus initially identified by comparing their mobilities to those of standard glycosaminoglycans. Subsequent quantitation of each glycosaminoglycan and its contribution to the overall glycosaminoglycan profile of each tissue was achieved by densitometric scanning.

This chapter now describes electrophoretic evidence which identifies and quantitates the constituent glycosaminoglycans isolated from separated specimens of human gingival epithelium and connective tissue.

MATERIALS AND METHODS

Extraction of Glycosaminoglycans

Following separation of gingival epithelium and connective tissue, each tissue type was pooled separately in acetone at 4°C until sufficient material was available for analysis. In general, 1-2 gm dry weight was sufficient for all glycosaminoglycan preparative studies. The pooled tissue was then placed in chloroform-methanol (2:1 v/v) for 2 days at 4°C. The resultant dry, defatted tissue was then weighed and subjected to the following proteolytic digestion to release the glycosaminoglycans.

Pooled gingival tissue specimens (approximately 1-2 gm) were digested in 20 ml of papain E.C. 3.4.22.2. (B.D.H. Chemicals) 1 mg/ml in 0.2 M sodium acetate buffer, pH 5.7, containing 0.004 M EDTA and 0.02 M Cysteine HCl (Sigma Chemicals, Sydney, Australia) at 60°C for 24 hours.

Following digestion, 80% trichloroacetic acid (w/v) was added to give a final concentration of 10%. After 1 hour at 4°C, the precipitated protein was centrifuged at 1000g for 15 minutes. The resultant pellet was then washed with 5% (w/v) trichloroacetic acid and recentrifuged (1000g). The supernatants were pooled and exhaustively dialysed against several changes of 0.05 M NaCl for 3 days. The retentate was then concentrated by rotary evaporation and adjusted to a known small volume (approximately one-tenth of the original volume).

The glycosaminoglycans were precipitated from this solution by the addition of 4 volumes of 1% sodium acetate in absolute ethanol (w/v). After 24 hours at 4°C, the precipitated glycosaminoglycans were centrifuged at 1000g for 15 minutes, washed with absolute ethanol and dried over phosphorous pentoxide *in vacuo*. The recovered glycosaminoglycans were then redissolved in 1 ml distilled water and aliquots taken for further analysis.

Electrophoresis of Extracted Glycosaminoglycans

Preliminary investigations to find a suitable electrophoretic system were carried out using standard glycosaminoglycan samples of hyaluronic acid, heparan sulphate, dermatan sulphate, chondroitin sulphate 4 and chondroitin sulphate 6. These were generously donated by Dr. M.B. Mathews (Chicago, Illinois, U.S.A.). The following is a description of the system which provided the most satisfactory separation of these glycosaminoglycans.

Standard glycosaminoglycan samples (3 µl) containing a mixture of 1 mg/ml of hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphates 4 and 6 were elctrophoresed simultaneously with a 5 µl sample of the glycosaminoglycans (1 mg/ml) extracted from both gingival epithelium and from connective tissue on Cellogel cellulose acetate membrane electrophoresis strips (Chemetron, Milan, Italy) in a Shandon electrophoresis chamber (Model 618, Shandon Products, England). The strips had been previously soaked for at least 30 minutes in 0.2 M calcium acetate buffer, pH 7.2, lightly blotted between filter paper and the samples applied at the cathode in a 0.5 cm line. The strips were placed in the electrophoresis tank and electrophoresed at 30 volt/cm for 30 minutes. To avoid excess heating and evaporation, they were covered with a layer of hydrophobic liquid (Ondina 15 medicinal oil, Shell Co.). After completion of electrophoresis, the strips were immersed in absolute ethanol for 5 minutes, stained and destained according to the method of NEWTON, SCOTT and WHITEMAN (1974) by placing them in a staining solution containing 0.2% Alcian Blue (Gurr, London, U.K.), in 0.05 M $MgCl_2$ and 0.025 M sodium acetate buffer, pH 5.8 in 50% ethanol-water and allowed to stand for 40 minutes at room temperature. The strips were then washed in three changes of solution containing 0.05 M $MgCl_2$, 0.025 M sodium acetate and 50% (v/v) ethanol-water, each for 30 minutes duration.

Clearing of the strips in preparation for subsequent densitometric scanning on a Canalco J 11 densitometer was achieved first by dehydrating in methanol for 1 minute followed by dry heating at 60°C for 5 minutes on a glass plate.

Confirmation of Glycosaminoglycan Species by Enzymatic and Chemical Means

a) Digestion with Streptomyces hyaluronidase

Standard and test samples containing 100 µg of uronic acid were digested in 0.5 M sodium acetate buffer, pH 5.0, containing 20 TRU/ml of Streptomyces hyaluronidase, E.C. 4.2.2.1. (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) at 60°C for 1 hour (OHYA and KANEKO, 1970). This enzyme is specific for hyaluronic acid. Various types of chondroitin sulphates, heparan sulphate and dermatan sulphate are not degraded by Streptomyces hyaluronidase.

The reaction solution was then deproteinized with trichloroacetic acid at a final concentration of 10%, allowed to stand overnight at 4°C and then centrifuged at 1000g. The supernatant was dialysed against water and the retentate lyophilized and reconstituted to a known small volume with distilled water. Samples of 5 µl were then subjected to electrophoresis as previously described.

b) Digestion with Chondroitinase AC II

Similarly, standard and gingival tissue glycosaminoglycan samples containing 100 µg of chondroitin sulphate were digested with chondroitinase AC II (Seikagaku Kogyo Co. Ltd., Tokyo) according to the method of SAITO, YAMAGATA and SUZUKI (1968). The enzyme is specific for chondroitin sulphates 4 and 6 and has no action on hyaluronic acid

heparan sulphate and dermatan sulphate. Essentially, the digestion involved incubation of the glycosaminoglycan samples in enriched Tris buffer (3 gm Tris HCl; 2.4 gm sodium acetate; 1.46 gm NaCl and 50 mg crystalline Bovine Serum Albumin in 100 ml 0.13 M HCl, pH 8.0) containing 0.3 units of chondroitinase AC II at 37°C for 30 minutes.

The resultant unsaturated disaccharides were applied to a Whatman No. 1 filter paper (7 cm x 56 cm). Descending paper chromatography was carried out as described by MURATA and BJELLE (1977) at room temperature. Desalting chromatography was performed in n-butanol-ethanol-water (52:32:16 v/v) for 18 hours and subsequent chromatography carried out in 1-butyric acid-0.5 M ammonia (5:3 v/v) for 20 hours. After drying the paper, the separated disaccharides were visualised with a Mineralight, Model S-2537 (Ultraviolet Products Inc., San Gabriel, California).

Following visualization, each spot was marked, cut out and eluted in 2 ml of distilled water overnight. Uronic acid analyses on 0.2 ml aliquots were performed as described previously.

c) Chemical Degradation of Glycosaminoglycans

Nitrous acid degradation for the detection of glycosaminoglycans containing N-sulphated groups was carried out according to the method of LAGUNOFF and WARREN (1962). Standard and gingival tissue glycosaminoglycan specimens containing hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate were dissolved in 0.8 ml of water, 0.1 ml of glacial acetic acid and 0.1 ml of 18% sodium nitrite. This was mixed and allowed to stand at room temperature for 90 minutes with occasional shaking. The reaction mixture was then lyophilized and reconstituted to a known small volume from which 5 µl samples were subjected to electrophoresis as previously described.

Glycosaminoglycan Quantitation

Glycosaminoglycans extracted from various tissue sources may be quantitated by integration of densitometric scans of cleared cellulose acetate membranes following electrophoresis (BREEN, et al., 1970). However, since each glycosaminoglycan has a different coefficient of binding for Alcian Blue, accurate quantitative values cannot be obtained by directly reading the integration values. Rather, calibration curves for each individual glycosaminoglycan relating the amount of Alcian Blue binding and glycosaminoglycan concentration must be obtained. Samples of standard glycosaminoglycan (3 µl) ranging in concentration from 0.1 mg/ml to 1.5 mg/ml were subjected to electrophoresis, staining and clearing as described above. The cleared strips were then scanned with a densitometer and an integrated value obtained for each glycosaminoglycan concentration. Thus, each integrated value represented the amount of Alcian Blue bound for each individual glycosaminoglycan. Equations for the slope of each line together with the correlation coefficient were obtained from four separate runs at each concentration for the individual glycosaminoglycan species.

This provided a means whereby tissue glycosaminoglycans could be quantitated. Integrated values of extracted gingival glycosaminoglycans could be read directly from these standard curves to provide the appropriate concentrations of glycosaminoglycan species in each specimen.

RESULTS

Tissue Separation

Accurate and meaningful observations on the macromolecular components of gingival epithelium and connective tissue can only

be made if these two closely apposed tissues are separated completely.

Excised gingivae incubated in phosphate buffered saline containing EDTA were easily separated into gingival epithelium and connective tissue using fine dissecting forceps. Histological sections of both human and cadaver specimens treated and separated in such a manner are shown in Figures 3.1 and 3.2. Two important features are revealed:

(a) Separation is complete along the basement membrane with no contamination of the epithelium by the connective tissue or vice versa. Separation is at the level of the lamina lucida of the basal lamina with an intact lamina densa covering the free surface of the connective tissue (SCALETTA and MAC CALLUM, 1972; McKENZIE, DABELSTEIN and ROED-PETERSON, 1979),

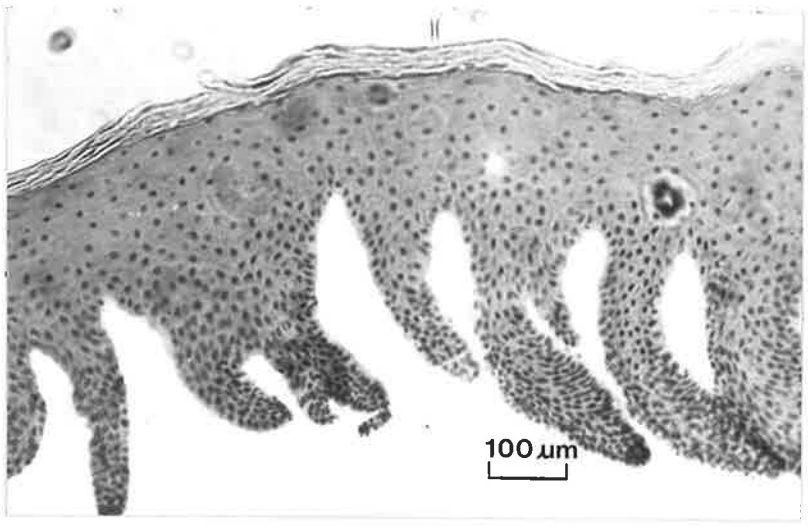
(b) The tissues appear to be structurally intact and relatively unaffected by the short incubation-separation procedure.

Furthermore, Figure 3.2 indicates few discernable differences at the light microscope level between the appearance of the tissues excised from cadavers and those from patients receiving periodontal therapy. A notable feature is the absence of inflammation in either of the tissue specimens.

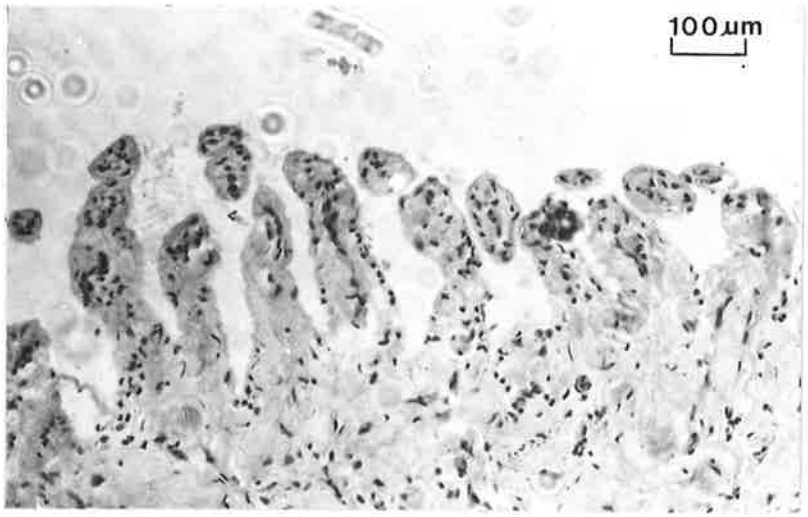
Assays for uronic acid in the EDTA-phosphate buffered saline used during incubation of gingivae were negative, indicating an insignificant loss of proteoglycans or glycosaminoglycans at this stage of the tissue preparation.

Glycosaminoglycan Extraction

The glycosaminoglycans of human gingival epithelium and connective tissue were recovered following papain digestion.



a



b

Figure 3.1

Light micrographs of human gingival tissue excised from a patient receiving periodontal treatment separated following incubation in phosphate buffered saline containing 10 mM EDTA. Sections 6 µm thick were cut and stained with Haematoxylin and Eosin (125X)

a) Epithelium

b) Connective Tissue

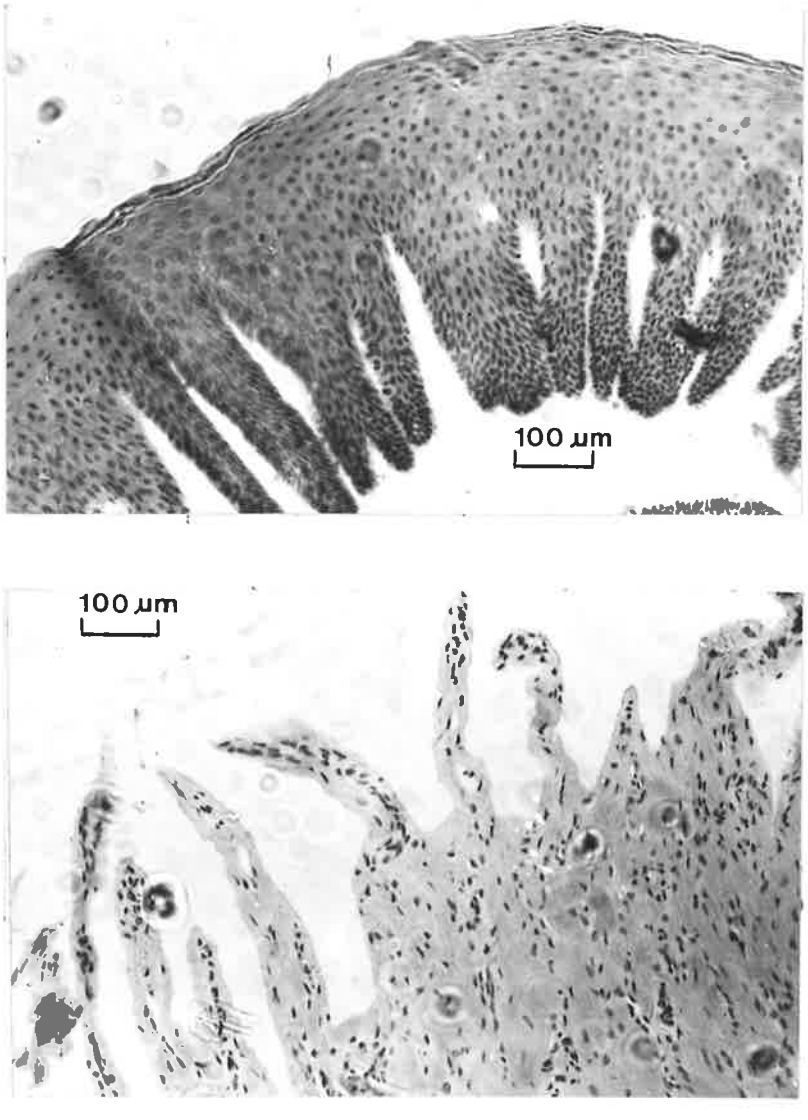


Figure 3.2

Light micrographs of human gingival tissue excised at autopsy on a human cadaver (27 year old male) and separated following incubation in phosphate buffered saline containing 10 mM EDTA. Sections 6 µm thick were cut and stained with Haematoxylin and Eosin (125X).

a) Epithelium

b) Connective Tissue

A typical profile of the amounts of uronic acid in the extracted glycosaminoglycans is listed in Table 3.1; uronic acid amounted to 0.07% and 0.23% respectively of the dry weights of gingival epithelium and connective tissue.

Galactose levels (used as an indicator of the presence of keratan sulphate) were negative for both tissue digests. It was therefore concluded that keratan sulphate was not a constituent of the gingival glycosaminoglycans.

Qualitative Identification of Glycosaminoglycans

The elctrophoretic separation of standard glycosaminoglycan samples of hyaluronic acid, heparan sulphate, dermatan sulphate, chondroitin sulphate 4 and chondroitin sulphate 6 is seen in Figure 3.3. The separation of these glycosaminoglycans was very good when they were electrophoresed individually; however, when electrophoresed as a mixture, chondroitin sulphates 4 and 6 did not separate clearly.

Electrophoresis of glycosaminoglycans isolated from gingival epithelium and connective tissue revealed three discrete regions of Alcian Blue affinity and corresponded to hyaluronic acid/heparan sulphate (1.0 cm/½hr); dermatan sulphate (1.55 cm/½hr); and chondroitin sulphate 4/ chondroitin sulphate 6 (2.15 cm/½hr). Although some separation within both the hyaluronic acid/heparan sulphate and chondroitin sulphate 4/chondroitin sulphate 6 regions appears probable, these bands were generally indistinct and quantitation was not reliable (Figure 3.4).

Table 3.1

Glycosaminoglycan Recovery from Human Gingival Epithelium and Connective Tissue

	Epithelium	Connective Tissue
Dry weight of tissue	177.31 mg	514.22 mg
Uronic acid recovered	0.131 mg	1.207 mg
Uronic acid/Dry weight	0.07%	0.23%

An example of the amount of glycosaminoglycan extracted from human gingivae following papain digestion. The dry tissue was first weighed and then subjected to papain digestion. Following recovery of the glycosaminolgycans by ethanol precipitation, the uronic acid levels were determined. The amount of glycosaminoglycan in epithelium and connective tissue was thus estimated and expressed as a percentage of uronic acid per mg dry weight.

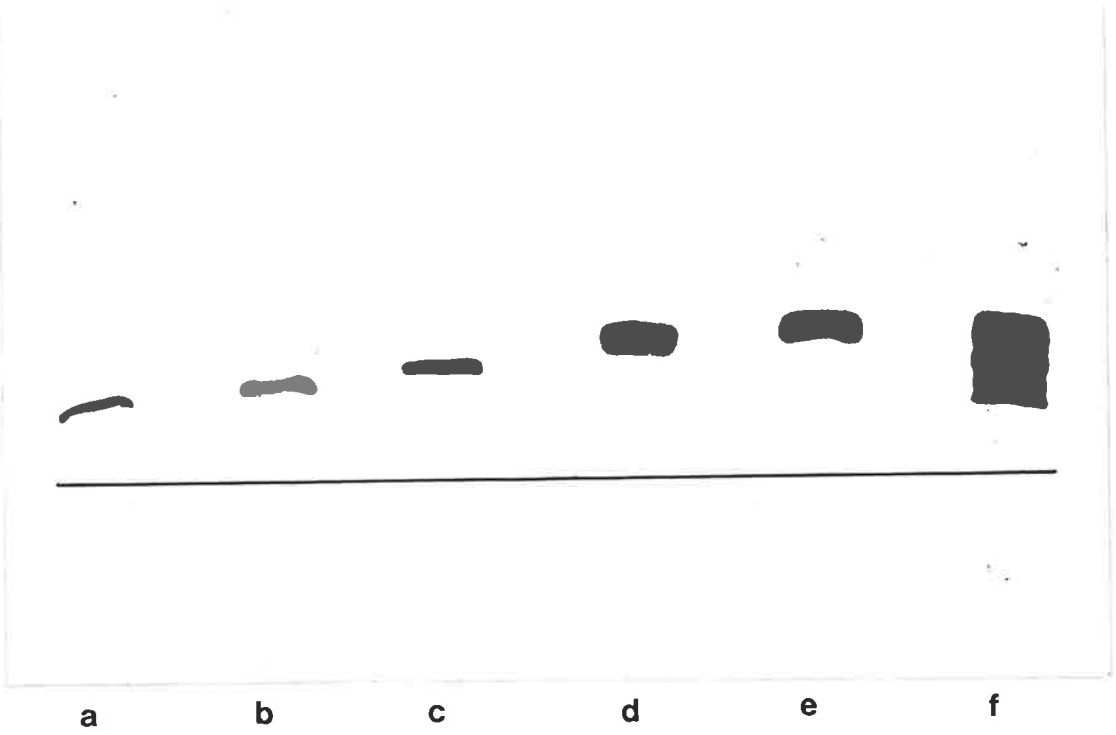


Figure 3.3

Electrophoresis of standard glycosaminoglycans. 3 µl samples were applied in a 0.5 cm line on cellulose acetate strips and electrophoresed at 30 volt/cm in 0.2 M calcium acetate. The strips were stained with 0.2% Alcian Blue.

a) Hyaluronic Acid

b) Heparan Sulphate

c) Dermatan Sulphate

d) Chondroitin Sulphate 4

e) Chondroitin Sulphate 6

f) Mixture

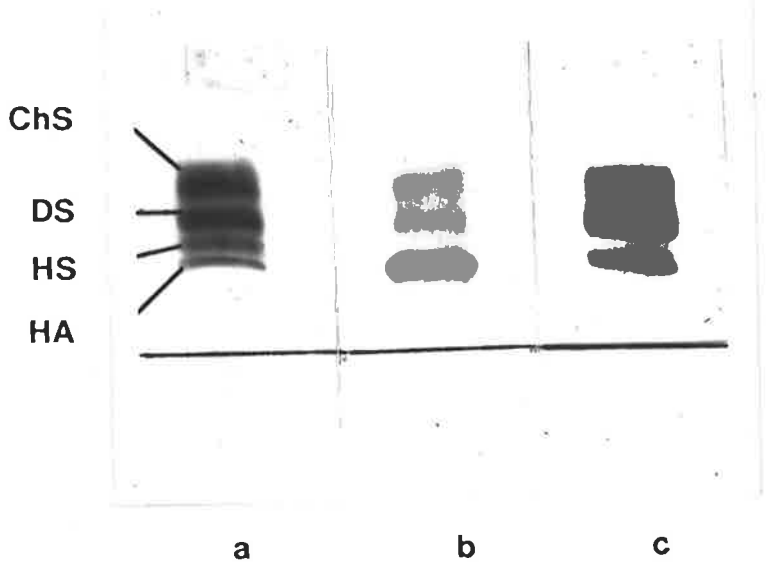


Figure 3.4

Electrophoretograms of:

a) Standard Glycosaminoglycans

b) Epithelial Glycosaminoglycans

c) Connective Tissue Glycosaminoglycans

The conditions for electrophoresis were the same as for Figure 3.3 Abbreviations: HA (hyaluronic acid); HS (heparan sulphate); DS (dermatan sulphate); ChS (chondroitin sulphate 4).

In order to confirm the identity of the glycosaminoglycans in each tissue, the polysaccharides were subjected to selective enzymatic and chemical degradation procedures.

Digestion of isolated glycosaminoglycan samples with Streptomyces hyaluronidase and subsequent electrophoresis revealed material in the hyaluronic acid/heparan sulphate region together with material corresponding to dermatan sulphate and chondroitin sulphate 4 or chondroitin sulphate 6 (Figure 3.5). Further treatment of hyaluronidase treated gingival glycosaminoglycans with nitrous acid and subsequent electrophoresis revealed an absence of Alcian Blue positive material in the hyaluronic acid/heparan sulphate region, whilst glycosaminoglycans corresponding to dermatan sulphate and chondroitin sulphate 4 or chondroitin sulphate 6 were still evident (Figure 3.5).

Figure 3.6 shows the electrophoretic separation of gingival epithelial and connective tissue glycosaminoglycan products following nitrous acid degradation. A band in the hyaluronic acid/heparan sulphate region together with dermatan sulphate and chondroitin sulphate 4 or chondroitin sulphate 6 can be seen. Further degradation of these glycosaminoglycans with Streptomyces hyaluronidase and subsequent electrophoresis resulted in no material being observed in the hyaluronic acid/heparan sulphate region (Figure 3.6).

Therefore, it was concluded that both hyaluronic acid and heparan sulphate were components of the indistinct hyaluronic acid/heparan sulphate region of epithelial and connective tissue digests noted in Figure 3.4.

Treatment of the isolated gingival glycosaminoglycans with chondroitinase AC II released the disaccharide units of chondroitin sulphate.

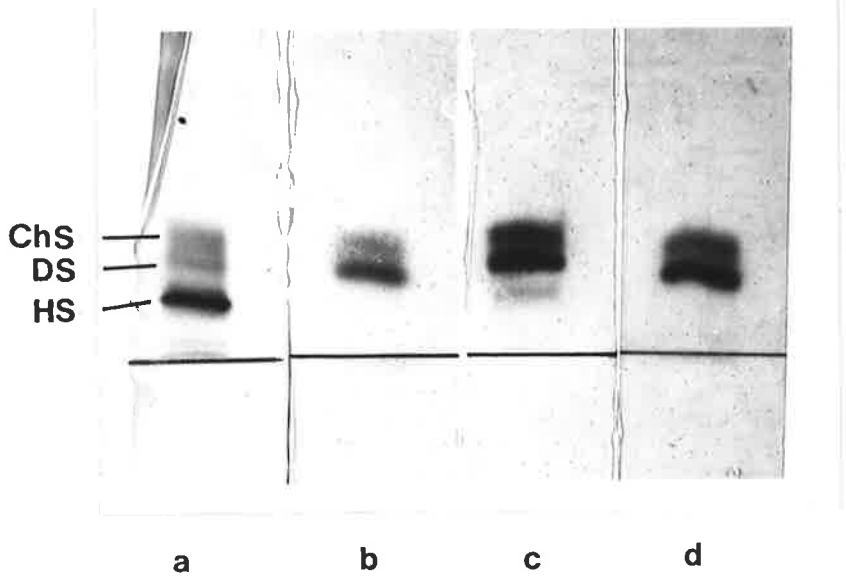


Figure 3.5

Electrophoretograms of gingival glycosaminoglycans treated first with Streptomyces hyaluronidase followed by nitrous acid.

a) Epithelial glycosaminoglycans treated with Streptomyces hyaluronidase.

b) Epithelial glycosaminoglycans treated with Streptomyces hyaluronidase and nitrous acid.

c) Connective tissue glycosaminoglycans treated with Streptomyces hyaluronidase.

d) Connective tissue glycosaminoglycans treated with Streptomyces hyaluronidase and nitrous acid.

The conditions for electrophoresis were the same as for Figure 3.3.
Abbreviations: HA (hyaluronic acid); HS (heparan sulphate); DS (dermatan sulphate); ChS (chondroitin sulphate 4).

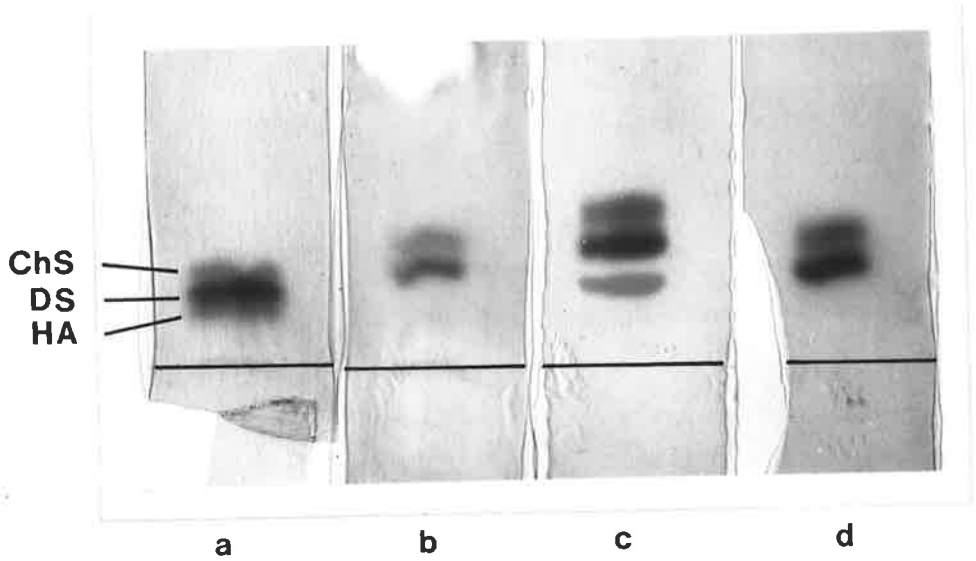


Figure 3.6

Electrophoretograms of gingival glycosaminoglycans treated first with nitrous acid followed by Streptomyces hyaluronidase.

a) Epithelial glycosaminoglycans treated with nitrous acid.

b) Epithelial glycosaminoglycans treated with nitrous acid and Streptomyces hyaluronidase.

c) Connective tissue glycosaminoglycans treated with nitrous acid.

d) Connective tissue glycosaminoglycans treated with nitrous acid and Streptomyces hyaluronidase.

The conditions for electrophoresis were the same as for Figure 3.3.

Abbreviations: HA (hyaluronic acid); HS (heparan sulphate); DS (dermatan sulphate); ChS (chondroitin sulphate 4).

Treatment of the isolated gingival glycosaminoglycans with chondroitinase AC II released the disaccharide units of chondroitin sulphate. These reaction products could be identified by paper chromatography and thus permitted the identification of the components comprising the chondroitin sulphate 4/chondroitin sulphate 6 band seen in Figure 3.4. The results of descending paper chromatography following chondroitinase AC II digestion of gingival epithelial and connective tissue glycosaminoglycans as well as standard samples of chondroitin sulphate 4 and chondroitin sulphate 6 are shown in Figure 3.7. A single spot corresponding to the reaction product of chondroitin sulphate 4 (2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-sulpho-D-galactose; designated ΔDi-4S) was observed following digestion of gingival glycosaminoglycans. This indicated that the chondroitin sulphate component of the chondroitin sulphate 4/chondroitin sulphate 6 band seen in Figure 3.4 is sulphated at position C-4. These spots on the paper chromatogram were marked with a pencil and cut out. Uronic acid analysis on overnight elutions from the excised spots revealed recoveries of 78% and 84% respectively for epithelial and connective tissue chondroitin sulphate.

Unsaturated O-sulphated disaccharides, probably derived from hyaluronic acid, were also observed following chondroitinase AC II digestion. However, these regions were not marked nor cut out for further analysis.

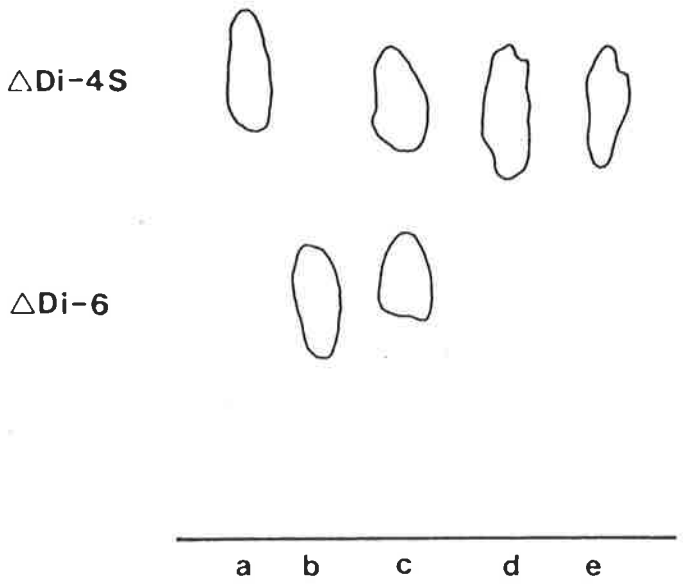


Figure 3.7

Tracing of descending paper chromatography of the reaction products of standard samples of chondroitin sulphate 4 and chondroitin sulphate 6 and gingival glycosaminoglycans digested by chondroitinase AC II. Descending paper chromatography performed in (i) n-butanol-ethanol-water (52:32:16 v/v) for 18 hours and (ii) 1-butyric acid-0.5 M ammonia (5:3 v/v) for 20 hours. Reaction products from:

a) Standard chondroitin sulphate 4
b) Standard chondroitin sulphate 6
c) Mixture of standard chondroitin sulphate 4 and chondroitin sulphate 6.
d) Epithelial glycosaminoglycans
e) Connective tissue glycosaminoglycans.

Quantitation of Glycosaminoglycans

Quantitative analysis of gingival glycosaminoglycans were also studied by integration of densitometric scans of cellulose acetate membranes following electrophoresis.

However, since various glycosaminoglycans appear to have different coefficients of binding for Alcian Blue, calibration curves plotting Alcian Blue absorbtion against individual standard glycosaminoglycan concentrations for hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 were prepared. In all cases, the curves demonstrated absolute linearity but their slopes varied (Figure 3.8). This confirmed that the amount of Alcian Blue bound per mole of hexosamine was different for each glycosaminoglycan. The equations for the slope of each line together with their correlation coefficients are seen in Table 3.2 and were used for direct calculation of tissue glycosaminoglycan levels.

As seen earlier in Figures 3.4 to 3.6, there are obvious differences in the distribution of the various glycosaminoglycan species of human gingival epithelium and connective tissue. Data compiled from readings taken from the integrations of densitometric scans of electrophoresed gingival glycosaminoglycans and adjusted for Alcian Blue binding are shown in Table 3.3. Quantitatively, the major differences noted were that heparan sulphate (59.6%) appeared to constitute the greatest proportion of the total epithelial glycosaminoglycan component. On the other hand, the greatest percentage of total connective tissue glycosaminoglycans was represented by dermatan sulphate (60.6%). In both tissues chondroitin sulphate 4 was the second largest contributor to the glycosaminoglycan profiles (20.1% in the epithelium and 28.04% in the connective tissue) whilst hyaluronic acid was present in both

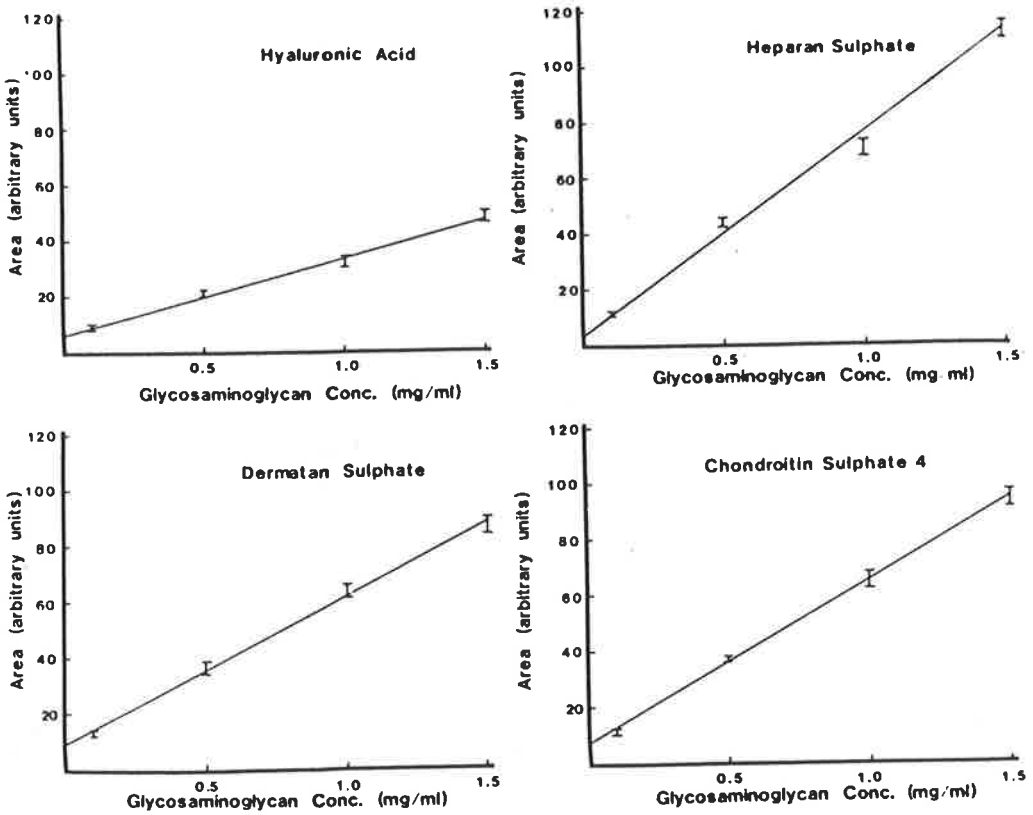


Figure 3.8

The relationship between standard glycosaminoglycan concentration and Alcian Blue affinity following electrophoresis on cellulose acetate membranes. Integrations of densitometric scans (which is a measure of the amount of Alcian Blue bound, and is expressed in arbitrary units of area) were plotted against the corresponding standard glycosaminoglycan concentrations.

Table 3.2

Slope Equations and Correlation Coefficients of Curves in Figure 3.8

Glycosaminoglycan	Slope	Correlation Coefficient
Hyaluronic Acid	y=7.73+25.51x	0.96
Heparan Sulphate	y=2.99+73.81x	0.96
Dermatan Sulphate	y=9.77+51.99x	0.99
Chondroitin Sulphate 4	y=7.48+57.69x	0.98

y=area

x=concentration of glycosaminoglycan

Four electrophoretic runs were made at each glycosaminoglycan concentration ranging from 0.5 mg/ml to 2.0 mg/ml. Three independent densitometric scans were made for each of the electrophoretic runs. The slope of each curve together with its correlation coefficient was calculated by continuous simple linear regression with an error correction routine.

Table 3.3

Glycosaminoglycan Content of Human Gingival Epithelium and Connective Tissue

Glycosaminoglycan	Epithelium		Connective Tissue	
	µg/gm dry wt.	%	µg/gm dry wt.	%
Hyaluronic Acid	125	5.2	126.4	3.58
Heparan Sulphate	1430	59.6	252.8	7.16
Dermatan Sulphate	358	14.9	2139.2	60.6
Chondroitin Sulphate	482	20.1	1011.2	28.04

The weight of each glycosaminoglycan species in human gingival epithelium and connective tissue was determined following densitometric scanning of electrophoretograms of the glycosaminoglycans. The amount of Alcian Blue associated with each glycosaminoglycan species (area of integrated densitometric scan) was substituted into the equations listed in Table 3.2. A value for the weight of glycosaminoglycan in each band on the basis of Alcian Blue binding was thus determined.

tissues in the smallest quantity (5.2% for the epithelium and 3.58% for the connective tissue). Neither chondroitin sulphate 6 nor keratan sulphate was observed in these studies.

DISCUSSION

A simple and effective technique for separating gingival epithelium from its underlying connective tissue has been adopted and slightly modified. The precision with which separation along the convoluted basement membrane could be achieved using a mild chelating agent was far superior to that obtained by simple microdissection. Consequently, for each tissue type the results can be reported with confidence as they reflect those of uncontaminated samples of epithelium and connective tissue.

Following routine digestion of tissues with papain and subsequent recovery of the glycosaminoglycans by ethanol precipitation, an electrophoretic separation, together with specific glycosaminoglycan elimination, was used to identify hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 as constituting the glycosaminoglycans of human gingival epithelium and connective tissue.

With the electrophoretic system used in this study, heparan sulphate appeared to move too closely with, and was sometimes superimposed on hyaluronic acid, thereby making identification and quantitation difficult. Nonetheless, the presence of heparan sulphate in both gingival epithelium and connective tissue has been confirmed following specific substrate elimination by enzymatic (*Streptomyces* hyaluronidase) and chemical (nitrous acid) degradation. Paper chromatography of the reaction products of chondroitinase AC II

identified chondroitin sulphate 4 as the constituent glycosaminoglycan of the chondroitin sulphate band.

The glycosaminoglycans extracted from human gingival epithelial and connective tissue in this study were further independently identified by courtesy of Dr. J.J. Hopwood employing a different electrophoresis system (HOPWOOD and HARRISON, 1982). Their method relies on sequential alcohol precipitation during the electrophoretic migration and was developed to specifically demonstrate heparan sulphate in urines of genetically deficient patients suffering from various forms of the mucopolysaccharidoses. With this method, confirmation of the presence of heparan sulphate and chondroitin sulphate 4 in gingival glycosaminoglycans was achieved. Furthermore, all four glycosaminoglycans (hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4) were demonstrated to be present in similar proportions as reported herein (see APPENDIX 4).

Of particular interest in the identification of human gingival epithelial and connective tissue glycosaminoglycans is the presence of heparan sulphate. Indeed, the heparan sulphate content of human gingivae has not been previously demonstrated. The fact that heparan sulphate has not been reported before is surprising since in the present study, this glycosaminoglycan appears to constitute the major glycosaminoglycan species of gingival epithelium (59.6%)

Several explanations are possible as to why heparan sulphate has not been noted previously. Firstly, no biochemical studies to date have considered gingival epithelium and connective tissue as separate organs; rather, previous studies have focused on whole gingival digests. Therefore, since epithelium is a relatively minor component of the gingivae with respect to its overall weight, the glycosaminoglycan component could have been masked by the more abundant connective tissue

constituents. Nonetheless, heparan sulphate has been demonstrated by this study to be present, albeit in small (7.16%) quantities, in connective tissue. Such a quantitatively small component may not have been identified in earlier studies because most of the glycosaminoglycan studies on human gingivae have relied essentially upon electrophoretic identification alone without subsequent enzymatic or chemical confirmation of the molecular species present.

The presence of heparan sulphate in human gingivae is, nevertheless, not a surprising finding because this glycosaminoglycan has been postulated to be an ubiquitous cell surface macromolecule (KRAEMER, 1972 a & b). Therefore, it would seem logical, on the basis of the high cellular density of epithelium, to find that heparan sulphate is the major contributor to the overall glycosaminoglycan composition of gingival epithelium.

By comparing the data obtained by densitometric scans of the electrophoresed gingival glycosaminoglycans, significant differences between the mobilities and relative amounts of glycosaminoglycans in gingival epithelium and connective tissue were noted. Whilst dermatan sulphate and chondroitin sulphate 4 are quantitatively major components of the epithelial glycosaminoglycans, they do not contribute quantitatively as much to the total glycosaminoglycan composition of epithelium as the connective tissue dermatan sulphate and chondroitin sulphate 4 species do to the overall glycosaminoglycan profile of connective tissue. Indeed, the major glycosaminoglycan component of the less cellular, more fibrous connective tissue is represented by dermatan sulphate and is consistent with the functional association of dermatan sulphate with collagen (ÖBRINK, 1973 a & b). The absence of both chondroitin sulphate 6 and keratan sulphate throughout

these analyses confirms the evidence obtained by analyses of whole human gingival tissues by EMBERY, OLIVER and STANBURY (1979) and on porcine gingiva by HIRAMATSU, ABE and MINAMI (1978).

The functional role of hyaluronic acid in gingival epithelium is difficult to explain if its presence should be related solely to a specific interaction with proteoglycans (WIEBKIN, BARTOLD and THONARD, 1979) similar to that described for cartilage and aorta (HARDINGHAM and MUIR, 1972; HASCALL and HEINEGÅRD, 1974; OEGEMA, HASCALL and EISENSTEIN, 1979). It is established (HARDINGHAM and MUIR, 1972; HASCALL, 1977) that only minute amounts of hyaluronic acid are necessary for proteoglycan/hyaluronic acid aggregate formation which may have importance in a structural capacity. It appears that there is more hyaluronic acid in the epithelial tissue than would be required for aggregate formation only. Therefore, the remaining non-interactive hyaluronic acid in the intercellular matrix could provide necessary viscoelastic properties, osmotic balance and other metabolic regulation factors required for healthy tissue function.

Indeed, as this study has elucidated the nature of the glycosaminoglycans of "normal" gingival epithelium and connective tissue, it would be of more than didactic interest to extend the findings to a consideration of the glycosaminoglycans of gingival tissues affected overtly by periodontal disease.

SUMMARY

The glycosaminoglycan components of the proteoglycans in the epithelial and connective tissue extracellular compartments of human gingivae have been extracted and analysed qualitatively as well as quantitatively.

Following proteolytic digestion of separated gingival epithelium and connective tissue, the glycosaminoglycans were recovered by ethanol precipitation. The total uronic acid content of these two tissue types amounted to 0.07% and 0.23% of the dry weight of epithelium and connective tissue respectively. They were identified electrophoretically and confirmation of the nature of the molecular species present was achieved by selective substrate elimination using Streptomyces hyaluronidase, chondroitinase AC II and nitrous acid degradation. The four regions demonstrating Alcian Blue affinity on cellulose acetate membranes following electrophoresis were thus identified as hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4. Neither chondroitin sulphate 6 nor keratan sulphate was observed.

Quantitatively, the major glycosaminoglycan component of the epithelial specimens was heparan sulphate (59.6%), whilst dermatan sulphate (60.6%) constituted the major glycosaminoglycan of human gingival connective tissue. Such differences are considered to reflect the general nature and function of these two closely apposed but structurally distinct tissues.

CHAPTER 4

MOLECULAR WEIGHT ESTIMATION OF THE SULPHATED GLYCOSAMINOGLYCANS IN HUMAN GINGIVAE

INTRODUCTION

The physiology of connective tissue is intimately related to the properties of the macromolecules constituting the extracellular matrix (LAURENT, 1968; COMPER and LAURENT, 1978). Therefore, since the biological properties of glycosaminoglycans are dependent upon the physicochemical parameters of these macromolecules, evidence of differing glycosaminoglycan molecular weights could be of particular significance in correlating functional properties.

To date, the many investigators who have isolated and studied the molecular weights of glycosaminoglycans associated with proteoglycans from a wide variety of tissues have adopted and developed many techniques.

Isolation and purification methods of glycosaminoglycans have included: sequential precipitation by ethanol (MEYER, et al., 1956; GARDELL, 1965); quaternary ammonium compounds (cetylpyridinium chloride and cetyltrimethylammonium bromide) in solution (SCOTT, 1965; SCHILLER, SLOVER and DORFMAN, 1961) and on inert supporting media, such as cellulose columns (GARDELL, 1965; ANTONOPOULOS, et al., 1964; SVEJCAR and ROBERTSON, 1967). Ion-exchange chromatography has also been used to separate the mammalian glycosaminoglycans (RINGERTZ and REICHARD, 1960; PEARCE, MATHIESON and GRIMMER, 1968).

Following separation of individual glycosaminoglycans, various methods for the determination of molecular weights have included gel chromatography, both for highly purified glycosaminoglycan reference samples (CONSTANTOPOULOS, DEKABAN and CARROLL, 1969; WASTESON, 1969) as well as for tissue extracts (RADHAKRISHNAMURTHY, et al., 1980), ultracentrifugation (PRESTON, 1968) and viscometry (RODÉN, et al., 1972). Polyacrylamide gel electrophoresis is another method which has been utilized (HILBORN and ANASTASSIADES, 1971; HSU, HOFFMAN and MASHBURN, 1973; MATHEWS and DECKER, 1971).

However, these studies have required relatively large quantities of material. In a tissue such as gingiva, where the quality of glycosaminoglycans is low and the availability of tissue for analysis is restricted, such techniques which lack sufficient sensitivity have limited usage. Indeed, attempts were made during the conceptual stages of the work described in this chapter to isolate individual glycosaminoglycan species present in human gingival epithelium and connective tissue. In particular, ethanol- and cetylpyridinium chloride-cellulose microcolumns as described by FRANSSON, et al. (1970) and SVEJCAR and ROBERTSON (1967) were tested. Various ion-exchange resins, including Whatman DE-32, Sephadex DEAE-A50 and Dowex AG 1-X2 Cl were investigated with respect to their suitability for separating mixtures of glycosaminoglycan species from gingiva. With these techniques, separation of non-sulphated glycosaminoglycans (hyaluronic acid) from the sulphated glycosaminoglycans (heparan sulphate, dermatan sulphate and chondroitin sulphate 4) was easily achieved. However, separation of the sulphated glycosaminoglycans from each other was not acceptable.

Therefore, a technique which could detect microgram quantities of glycosaminoglycans as well as permit clear-cut separation of each species was required. To achieve this, electrophoretic separation together with radiolabelled end group analysis following alkaline cleavage of glycosaminoglycans from proteoglycans was adopted and suitably modified.

The glycosaminoglycans were extracted from separated samples of gingival epithelium and connective tissue under alkaline conditions and end labelled in the presence of tritiated sodium borohydride simultaneously with the β-elimination reaction (ROBINSON and HOPWOOD,

1973; HOPWOOD and ROBINSON, 1973). Number-average molecular weight estimation was then carried out on samples of sulphated glycosaminoglycans separated by electrophoresis on cellulose acetate membranes.

The molecular weight of gingival hyaluronic acid could not be reliably determined by these methods due to the presence of apparently alkali labile regions along this molecule. This subject forms the basis of Chapter 5.

MATERIALS AND METHODS

Preparation of Tritiated Sodium Borohdride Solution

A stock alkaline solution of tritiated sodium borohydride (NaB^3H_4) was prepared at 4°C by dissolving 25 mCi (0.925 GBq) of NaB^3H_4 (Radiochemical Centre, Amersham, England) in 62.5 ml of 0.5 M KOH containing 0.2 M $NaBH_4$ (B.D.H. chemicals) and stored at -20°C. Prior to use, the alkaline NaB^3H_4 stock solution was diluted with 9 volumes of 0.5 M KOH to make 0.02 M borohydride solution in 0.5 M KOH.

Determination of Specific Activity of NaB^3H_4

The specific activity of the NaB^3H_4 was determined by reductive end labelling of benzophenone (Sigma Chemicals). To 1.3 ml of methanol, 5 mg of benzophenone was added and made up to 0.5 M KOH adding 1.3 ml of 1 M KOH. Approximately 0.3 ml of the NaB^3H_4 stock solution was then added and gently stirred at room temperature for 48 hours. The reaction was stopped by acidification to pH 5.0 with glacial acetic acid. The solution was then rotary evaporated to dryness. Crystal formation was induced by the addition of 2.5 ml distilled water. The resultant crystals were extracted into ether and evaporated to dryness. The

residue was redissolved in methanol and an aliquot taken for determination of radioactivity in a Packard Tri-Carb liquid scintillation spectrophotometer (Model 2405). Quench corrections were determined using the machine's automatic external standard and a set of Packard quenched standards (Packard Instrument Co. Inc. U.S.A.) (see APPENDIX 5).

Quantitation of the recovered, reduced benzophenone was achieved by reading its absorbance at 259 nm. Reduced benzophenone had an extinction coefficient of 408 at 259 nm. The stages of ether extraction followed by drying, redissolving in methanol, tritium counting and quantitation were repeated until a constant specific activity was obtained. The specific activity of the NaB^3H_4 was finally determined as 6.03×10^5 d.p.m./µMole.

Extraction and [^{3}H]-Labelling of Gingival Glycosaminoglycans

Gingival connective tissue (1.75 mg) was placed into 50 ml of dilute (0.02 M) NaB^3H_4 solution and gently stirred at 4^oC for 11 days. The residual tissue remaining after this treatment was separated from the borohydride solution by centrifugation (1000g). The supernatant was acidified by adding glacial acetic acid to pH 5.0. This was allowed to stand for 1-2 hours because the acid caused the formation of a precipitate which was required to settle. Subsequent analysis of the precipitate for uronic acid (BLUMENKRANTZ and ASBOE-HANSEN, 1973) and protein (LOWRY, et al., 1951) revealed it to be proteinaceous and contained no uronic acid; this was discarded. Further elimination of protein was achieved by adding 80% trichloroacetic acid to a concentration of 10% and allowing this to stand at 4^oC for 1 hour. The resultant protein precipitate was removed by centrifugation (1000g) and the supernatant exhaustively dialysed against deionized water for 3 days. The retentate was then concentrated by rotary

evaporation and the glycosaminoglycans precipitated by adding 4 volumes of 1% (w/v) sodium acetate in ethanol and leaving overnight at 4°C. The precipitated glycosaminoglycans were isolated by centrifugation (1000g) and the supernatant discarded. Following drying over phosphorous pentoxide in vacuo, the precipitate was redissolved in a small volume of water (1 ml) and aliquots taken to determine the amount of uronic acid present.

The gingival connective tissue remaining after KOH extraction was digested by pure papain, E.C. 3.4.22.2. (B.D.H. Chemicals) to relase glycosaminoglycans not extracted by the alkaline hydrolysis. The remaining glycosaminoglycans were recovered by the methodology described in Chapter 3. The efficiency by which glycosaminoglycans were extracted by KOH from gingival connective tissue was thus determined.

Gingival epithelium (1.0 gm) was subjected to the same extraction and labelling procedure. However, in contrast to connective tissue, after 11 days at 4°C in the dilute NaB^3H_4 solution there was no residual tissue. Consequently, papain digestion of residual tissue following KOH extraction was not required for the epithelium.

[^{3}H] - Labelling of Standard Sulphated Glycosaminoglycans

In order to verify that the above technique was satisfactory for molecular weight estimations, standard samples of dermatan sulphate and chondroitin sulphate 4 were subjected to alkali reduction and end labelling by β-elimination in the presence of NaB^3H_4.

Samples of dermatan sulphate and chondroitin sulphate 4 (3 mg each) were dissolved separately in 10 ml of the diluted (0.02 M) NaB^3H_4

solution and gently stirred at 4°C for 11 days. Subsequent acidification to pH 5.0 with glacial acetic acid, dialysis, concentration and precipitation was carried out as for the gingival extractions, except that no trichloroacetic acid precipitation of protein was necessary.

Electrophoresis

A modification of the electrophoretic technique described in Chapter 3 was adopted which provided better separation of hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4.

Development of Improved Electrophoretic Separation

Before the technique of end labelling gingival glycosaminoglycans for molecular weight estimation could be used confidently, the electrophoretic separation of hyaluronic acid and heparan sulphate in 0.2 M calcium acetate (as used previously) required improvement. Whilst preliminary experiments were being carried out, three papers appeared in the literature describing a discontinuous electrophoretic system which separated all seven known mammalian glycosaminoglycans (CAPPELLETTI, DEL ROSSO and CHIARUGI, 1979 a & b, 1980). The technique, which involved sequential precipitation of glycosaminoglycans during electrophoresis, was tested using both standard and gingival glycosaminoglycans. Unfortunately, our apparatus could not be modified sufficiently to the standards specified by CAPPELLETTI, DEL ROSSO and CHIARUGI (1979 a) and consequently the electrophoretic results were disappointing.

Nonetheless, since only hyaluronic acid and heparan sulphate required improved separation in our previously described system (Chapter 3), the concept of combined electrophoresis and sequential ethanol precipitation was developed further.

On the basis that hyaluronic acid is more soluble in ethanol than heparan sulphate (MEYER, et al., 1956), the possibility of adding 17% ethanol (v/v) to the electrophoretic buffer and performing electrophoresis in 0.2 M calcium acetate containing 17% ethanol (v/v) was investigated. The technique was based on the assumption that heparan sulphate would be precipitated whilst hyaluronic acid should remain in solution. If this were so, then one might also expect dermatan sulphate to precipitate at the concentration of ethanol used (MEYER, et al., 1956). Therefore, the electrophoretic running times would need to be adjusted to avoid overlapping of hyaluronic acid on dermatan sulphate. A further consideration was the possibility that if both heparan sulphate and dermatan sulphate were completely precipitated by 17% ethanol (and therefore immobilized on the electrophoresis strip) neither would move from the origin and no separation would be achieved.

Fortunately, when put to the test, adquate separation of gingival heparan sulphate, hyaluronic acid and dermatan sulphate was achieved (see Results).

In effect, the method adopted for this series of experiments was as follows. Electrophoresis of standard and gingival glycosaminoglycans was performed on Cellogel cellulose acetate strips (2.5 cm x 7 cm) which had previously been soaked in 0.2 M calcium acetate, pH 7.2 containing 17% (v/v) ethanol. The glycosaminoglycan specimens (5 µl and 10 µl for gingival tissue extracts; 3 µl for standard samples) were applied to the strips and electrophoresed at 30 volts/cm

for 40 minutes in a Shandon electrophoresis chamber containing the same buffer (0.2 M calcium acetate, pH 7.2 containing 17% (v/v) ethanol) as used for equilibration of the strips. A layer of hydrophobic liquid (Ondina 15 Medicinal Oil) was used as a coolant to prevent excess evaporation from the strips, as described in Chapter 3.

The separated glycosaminoglycans were located by Alcian Blue staining and the strips subsequently destained and cleared as described in Chapter 3.

Confirmation of Glycosaminoglycan Species Extracted by KOH

Confirmation of the molecular species as hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 was achieved by the use of Streptomyces hyaluronidase, chondroitinase AC II and nitrous acid degradation as described in Chapter 3.

Quantitation of Glycosaminoglycans

The relative amounts of individual glycosaminoglycans were determined essentially as previously described (Chapter 3). Densitometric scans of cleared electrophoretic strips using a Canalco J11 densitometer provided integrated values of the staining intensity of the separated glycosaminoglycan species. Standard curves based on densitometry and integrated values of known standard electrophoresed glycosaminoglycan samples permitted calculation of the concentrations of glycosaminoglycans extracted from gingivae.

Liquid Scintillation Counting

The recovery of radioactively labelled material on various filters and supports has been investigated previously (BOLLUM, 1959, 1966; MANS and NOVELLI, 1961) and criticisms of poor recovery using these techniques have been refuted (SCHRIER and WILSON, 1973). In consequence, a similar technique to that described by BOLLUM (1966) for the measurement of tritiated DNA on nitrocellulose filters was applied to the measurement of tritium end labelled glycosaminoglycans isolated on cellulose acetate electrophoresis membranes.

Following staining with Alcian Blue, destaining, clearing and densitometric determination of the amount of each glycosaminoglycan present, the bands were cut out and placed in 1 ml 2-methoxy ethanol. This was shaken vigourously until the cellulose acetate membrane was completely solubilized. Once dissolved, 9 mls of liquid scintillation fluid containing 5 gm 2,5-diphenyloxazole (PPO) and 0.3 gm p-bis [2-(5-phenyloxazolyl)] benzene (POPOP) per litre of scintillation grade toluene was added to each vial. Counting for tritium was performed in a Packard Tri-Carb liquid scintillation counter. The efficiency for tritium was calculated as 50.65% using the automatic external standard. Corrections for quenching by cellulose acetate and Alcian Blue were made using the automatic external standard.

The number average molecular weight of each glycosaminoglycan was then determined by end group analysis. This method assumes one specific radioactive label per polysaccharide chain (ROBINSON and HOPWOOD, 1973) and a value can be derived by relating the amount of polysaccharide and the specific activity of the borohydride itself

to the [^{3}H] d.p.m. in each band. This is shown in equation 1.

$$\text{nber Average Molecular Weight} = \frac{\text{Weight of sample x specific activity of } NaB^3H_4}{\text{d.p.m. of sample}} \quad (1)$$

weight - calculated from desitometric scan

d.p.m. - calculated from c.p.m. reading of liquid scintillator

specific activity - 6.03×10^5 d.p.m. /μ M benzophenone

d.p.m. = decays per minute

c.p.m. = counts per minute

The proposed mechanism whereby glycosaminoglycan chains are cleaved from proteoglycan core protein by β-elimination and simultaneously end labelled in the presence of potassium hydroxide and tritiated sodium borohydride is shown in figure 4.1.

RESULTS

Glycosaminoglycan Extraction Under Alkaline Conditions

Following KOH/NaB^3H_4 treatment of gingival epithelium there was complete solubilization of the tissue. The amount of glycosaminoglycan extracted by this procedure was 0.08% of the epithelial dry weight.

However, following the same treatment of the connective tissue there was some residual tissue remaining. Therefore, in order to determine the efficiency of KOH/NaB^3H_4 in extracting gingival connective tissue glycosaminoglycans, the residual tissue was subsequently subjected to papain digestion and glycosaminoglycan extraction as described. Between 85-90% of the total uronic acid present in gingival connective tissue was extracted under the alkaline conditions with the remaining 10-15% being released following papain digestion (Table 4.1).

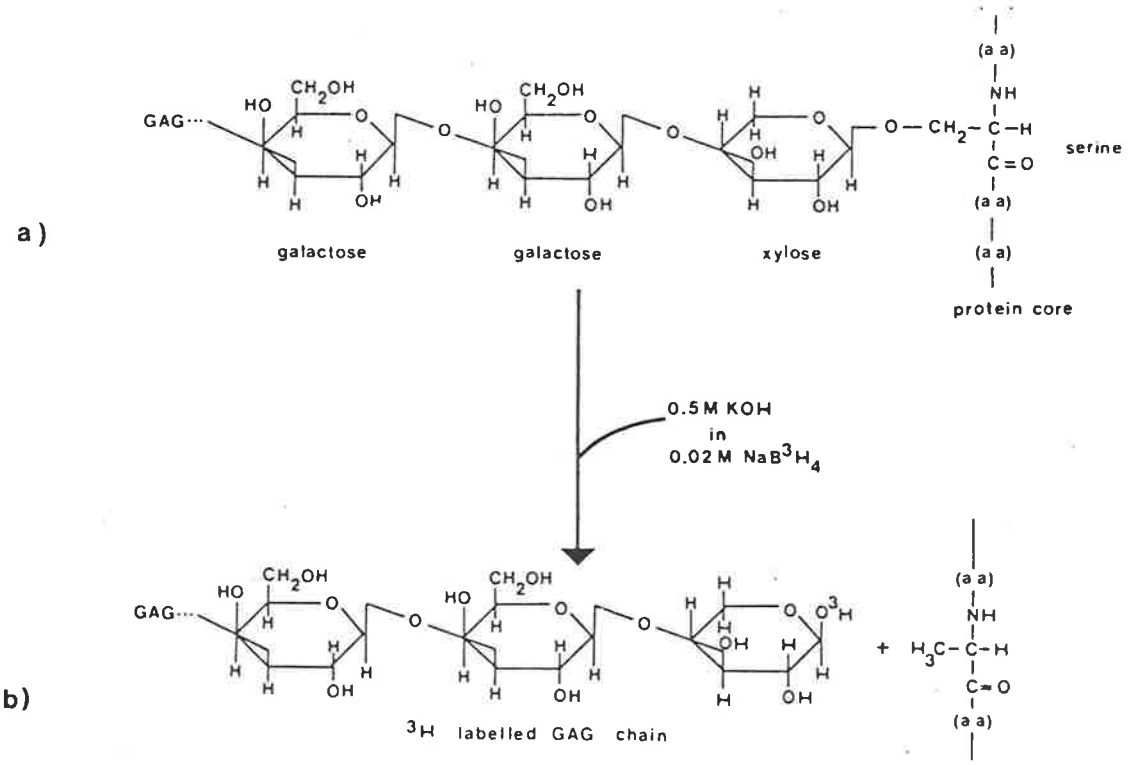


Figure 4.1

Schematic representation of cleavage and radiolabelling of glycosaminoglycans from proteoglycans by treatment with alkali in the presence of tritated sodium borohydride.

a) Association of glycosaminoglycans with proteoglycan core protein.

b) Effect of alkali cleavage and radiolabelling. The glycosidic bond between xylose and serine is broken and a tritium label appears on the hydroxyl group of C-1.

Table 4.1

Extraction of Connective Tissue Glycosaminoglycans

Treatment	Percentage of Total Glycosaminoglycans Extracted		
	Specimen A	Specimen B	Specimen C
Alkali	83%	90%	86%
Papain	17%	10%	14%

Glycosaminoglycans were extracted from three pools of connective tissue under alkaline conditions (0.5 M KOH) for 11 days. The residual tissue was then subjected to papain digestion. The relative amounts of total glycosaminoglycan removed from each of the three gingival connective tissue specimens are expressed as percentages of the total uronic acid extracted from the tissue.

Molecular Weight Estimation of Standard Sulphated Glycosaminoglycans

Using two standard sulphated glycosaminoglycan samples (dermatan sulphate and chondroitin sulphate 4) of known molecular weight (this information was supplied by courtesy of Dr. M.B. Mathews, Chicago; see APPENDIX 6), preliminary experiments were developed to establish the validity of using end group labelling, electrophoresis and densitometric quantitation for molecular weight estimation of microgram quantities of glycosaminoglycans. Molecular weights of 47,000 and 13,000 respectively were calculated for standards of dermatan sulphate and chondroitin sulphate respectively using this method. These are in good agreement with the independently determined molecular weight values supplied by Dr. M.B. Mathews (Table 4.2).

Separation of Glycosaminoglycans

a) Preliminary investigations

Early attempts to separate mixtures of glycosaminoglycan species were made using ion-exchange chromatographic techniques. In particular, Dowex 1-X2 Cl (as described by PEARCE, MATHIESON and GRIMMER, 1968), Whatman DE-32 and Sephadex DEAE-A50 were investigated. In all cases separation of the sulphated glycosaminoglycans (heparan sulphate, dermatan sulphate and chondroitin sulphate 4) was not obtained without some degree of contamination by another glycosaminoglycan species. Hyaluronic acid was the only glycosaminoglycan recovered free from other residual glycosaminoglycans (Figure 4.2).

The results shown are for sequential elutions only. However, similar results were also observed if these columns were eluted by a continuously increasing salt gradient. Sequential elution of similar mixtures of standard glycosaminoglycans on cetylpyridinium chloride-cellulose micro-columns (SVEJCAR and ROBERTSON, 1967) with increasing salt concentrations also proved unsatisfactory (Figure 4.3).

Table 4.2

Molecular Weight Estimation of Standard Glycosaminoglycans

Glycosaminoglycan	A Calculated Mol. Wt.	B Reported Mol. Wt.
Dermatan Sulphate	4.7×10^4 (± 0.08)	4.5×10^4
Chondroitin Sulphate 4	1.3×10^4 (± 0.07)	1.5×10^4

Comparison of (A) experimentally calculated molecular weight estimations for standard samples of dermatan sulphate and chondroitin sulphate 4 by end group labelling and quantitation following electrophoresis to (B) molecular weight values in the technical data sheet supplied with the standard glycosaminoglycan samples (see APPENDIX 6). The values in brackets are standard errors of the mean value.

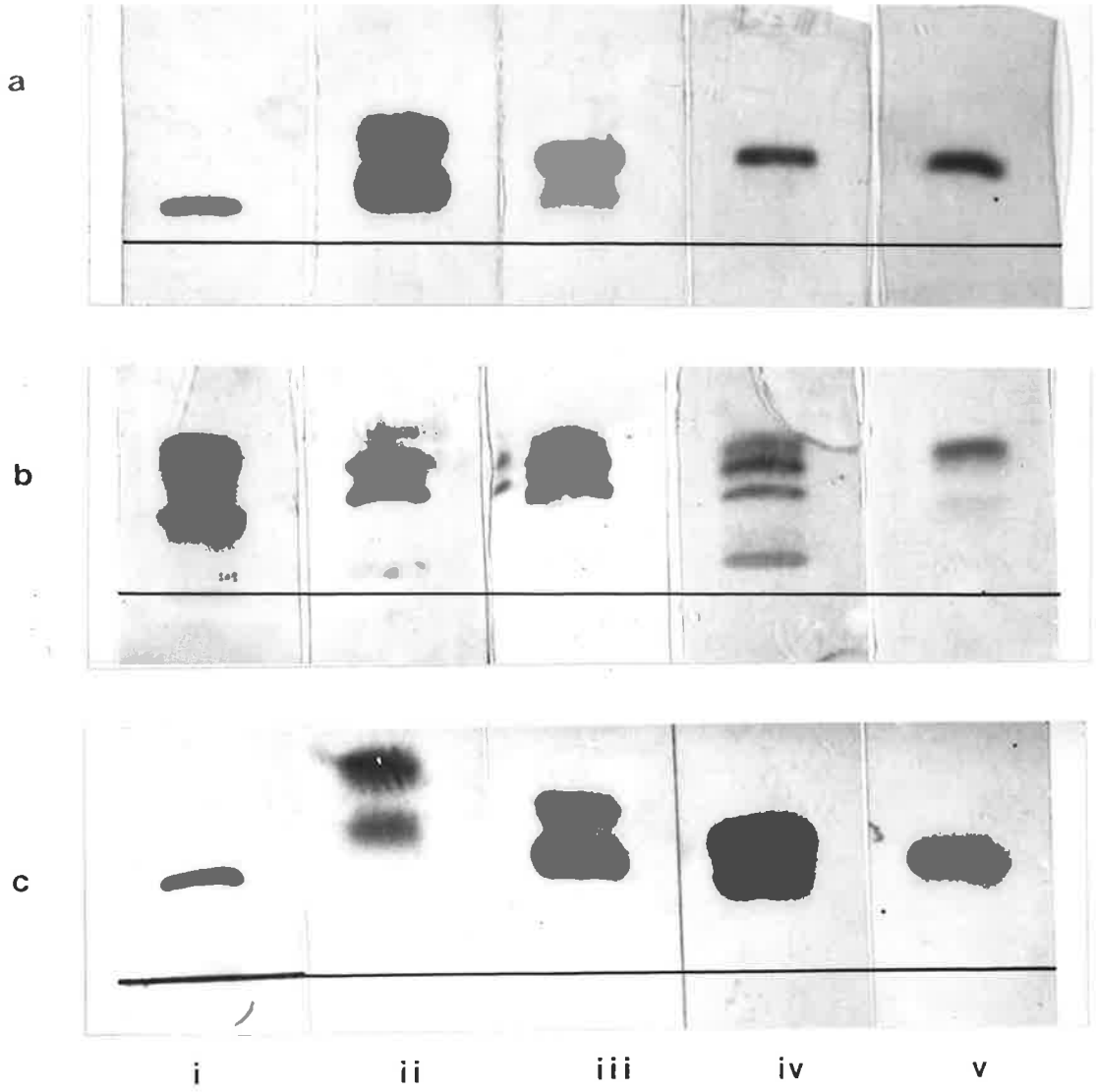


Figure 4.2

Electrophoretograms of the glycosaminoglycans obtained from stepwise elution of a mixture of glycosaminoglycans on (a) Dowex 1-X2 Cl (b) Whatman DE-32 and (c) Sephadex DEAE-A50 ion-exchange resins. The technique employed was essentially the same as that described by PEARCE, MATHIESON and GRIMMER (1968). Solutions containing equal proportions of hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 (1 mg/ml) were applied to columns containing one of the above ion-exchange resins. The resins had previously been equilibrated with 8 M urea, pH 7.0. The glycosaminoglycans were eluted stepwise with (i) 0.5 M NaCl; (ii) 1.25 M NaCl; (iii) 1.5 M NaCl; (iv) 1.75 M NaCl; (v) 2.0 M NaCl in the presence of 8 M urea. Whilst the individual glycosaminoglycan species are not marked it is clear that separation of individual glycosaminoglycans into single bands has not been achieved for most elutions.

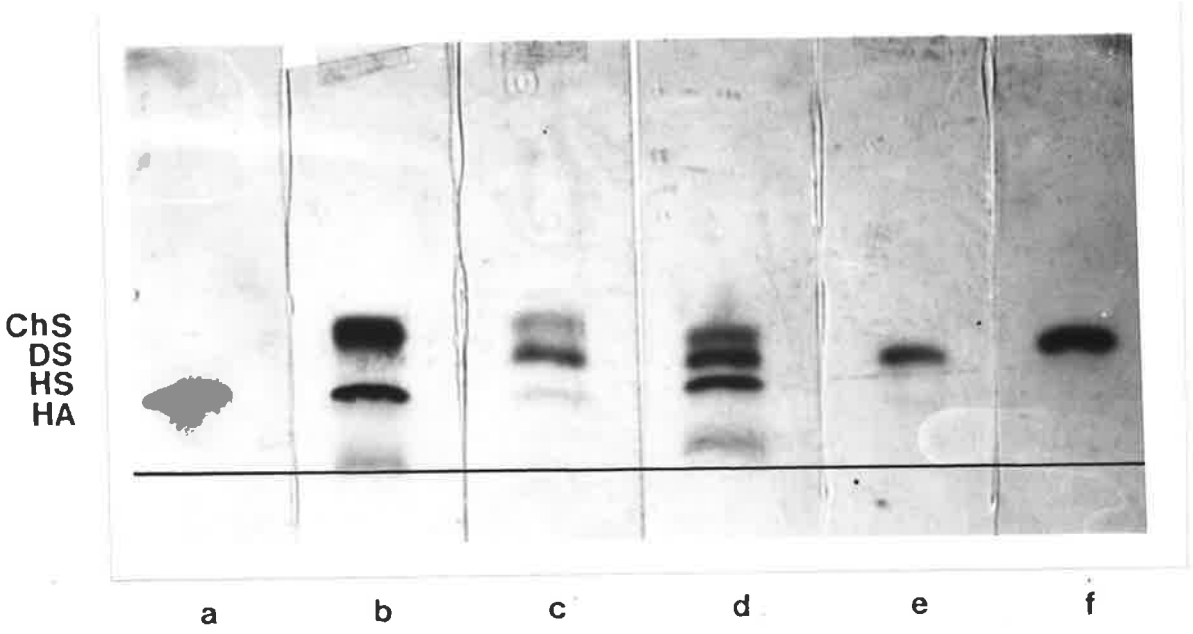


Figure 4.3

Electrophoretograms of attempts to separate a mixture of hyaluronic acid (HA); heparan sulphate (HS); dermatan sulphate (DS); and chondroitin sulphate 4 (ChS) on CPC-cellulose microcolumns.

The technique employed was the same as that described by SVEJCAR and ROBERTSON (1967). Glycosaminoglycan mixtures were applied to columns containing 10% cellulose suspended in 1% CPC and eluted stepwise with (a) 1% CPC; (b) 0.3 M NaCl; (c) 0.3 M $MgCl_2$; (d) 40% propanol-20% methanol-1.5% acetic acid; (e) 0.75 M $MgCl_2$-0.1 M acetic acid; (f) 0.75 M $MgCl_2$.

Such methods were therefore considered unsuitable for separating samples of hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 from gingival tissues.

b) Electrophoresis

Electrophoretic separation of standard samples of hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 individually or as a mixture in 0.2 M calcium acetate buffer pH 7.2 containing 17% ethanol (v/v) is shown in Figure 4.4. Under these conditions the mixture of four standard glycosaminoglycans separated into three bands only, with hyaluronic acid and heparan sulphate being superimposed. On the other hand, electrophoresis of the gingival glycosaminoglycans resulted in separation into four discrete bands which were identified following enzyme (Streptomyces hyaluronidase and chondroitinase AC II) and chemical (nitrous acid) degradation. Streptomyces hyaluronidase treatment removed the material comprising the band corresponding electrophoretically to hyaluronic acid, whilst nitrous acid treatment of the gingival glycosaminoglycans extracted under alkaline conditions degraded heparan sulphate. The presence of chondroitin sulphate 4 was confirmed by digestion of the glycosaminoglycans with chondrotinase AC II and subsequent paper chromatography (Figure 4.5).

As can be seen, the order of electrophoretic migration of gingival glycosaminoglycans in 0.2 M calcium acetate containing 17% ethanol is heparan sulphate (0.6 cm/30min.), hyaluronic acid (0.75 cm/30min.), dermatan sulphate (1.3 cm/30min.) and chondroitin sulphate 4 (1.5 cm/30min.). Since these rates are slower than those reported in Chapter 3, the electrophoretic running times were increased from 30 minutes to 40 minutes to obtain adequate separation.

Following densitometric scanning of cleared electrophoretic strips,

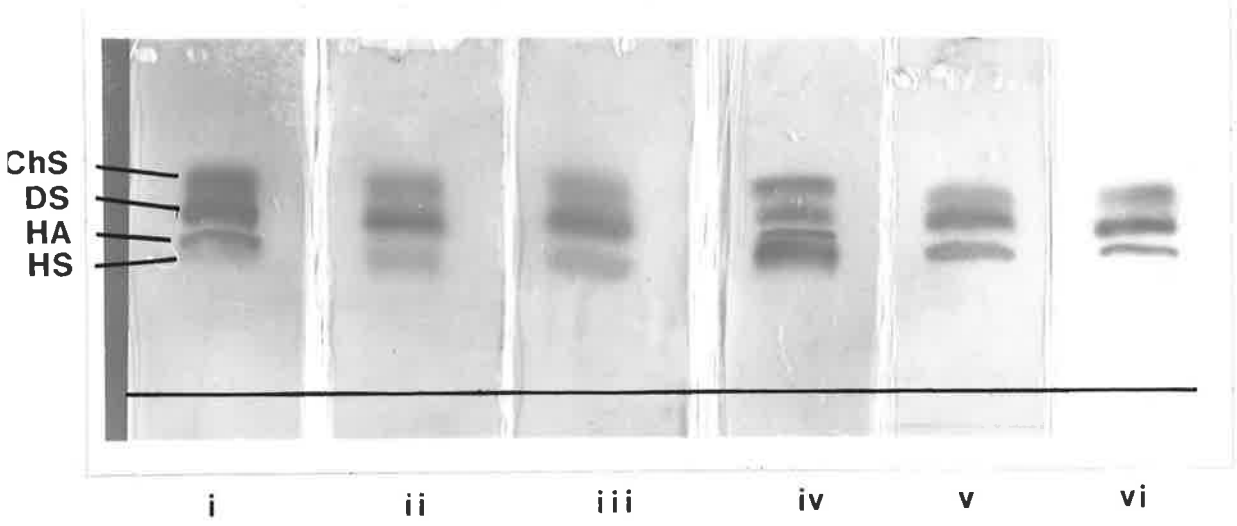


Figure 4.4

Electrophoretograms of (a) human gingival epithelial glycosaminoglycans and (b) human connective tissue glycosaminoglycans extracted under alkaline conditions (0.5 M KOH). Electrophoresis was performed in 0.2 M calcium acetate containing 17% (v/v) ethanol for 40 minutes at 30 volt/cm. The glycosaminoglycans were stained with 0.2% Alcian Blue. Abbreviations: HA (hyaluronic acid); HS (heparan sulphate); DS (dermatan sulphate); ChS (chondroitin sulphate 4).

(i) Epithelial glycosaminoglycans

(ii) Epithelial glycosaminoglycans previously treated with Streptomyces hyaluronidase.

(iii) Epithelial glycosaminoglycans previously treated with nitrous acid.

(iv) Connective tissue glycosaminoglycans.

(v) Connective tissue glycosaminoglycans treated with Streptomyces hyaluronidase.

(vi) Connective tissue glycosaminoglycans treated with nitrous acid.

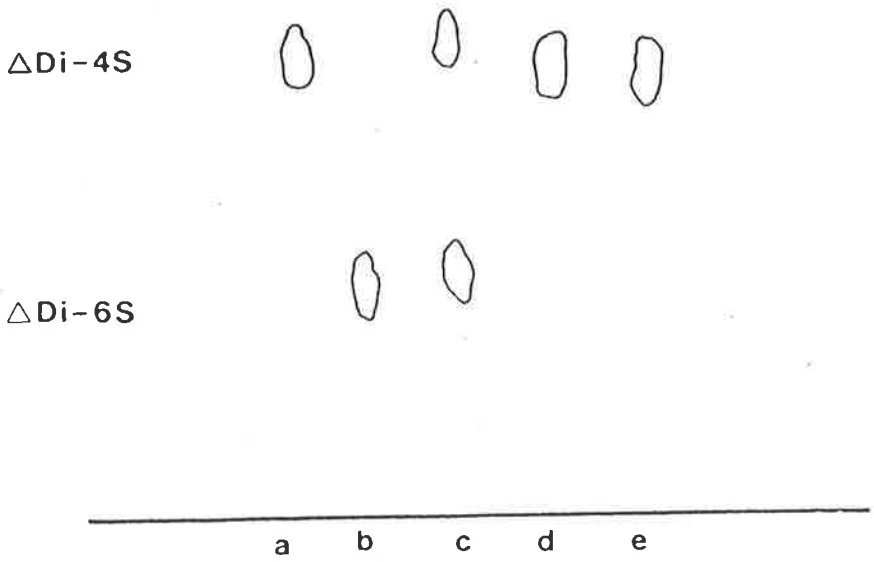


Figure 4.5

Tracing obtained from descending paper chromatography of the reaction products of standard samples of chondroitin sulphate 4 and chondroitin sulphate 6 and gingival glycosaminoglycans digested by chondroitinase AC II. Descending paper chromatography performed in (i) n-butanol-ethanol-water (52:32:16 v/v) for 18 hours and (ii) 1-butyric acid-0.5 M ammonia (5:3 v/v) for 20 hours. Reaction products from:

a) Standard chondroitin sulphate 4
b) Standard chondroitin sulphate 6
c) Mixture of standard chondroitin sulphate 4 and chondroitin sulphate 6.
d) Epithelial glycosaminoglycans
e) Connective tissue glycosaminoglycans.

the amount (weight) of each glycosaminoglycan in each Alcian Blue positive band was calculated. The relative proportions of individual glycosaminoglycans in gingival epithelium and connective tissue are shown in table 4.3. and are very similar to the proportions reported in Chapter 3 for glycosaminoglycans extracted by papain. Heparan sulphate was quantitatively the major epithelial glycosaminoglycan whilst dermatan sulphate predominated in the connective tissue glycosaminoglycans. In both tissues chondroitin sulphate 4 was quantitatively the second major glycosaminoglycan species. The bands were then carefully cut out and the amount of radioactive label present was determined for each electrophoretic band at either 5 µl and 10 µl gingival glycosaminoglycan loadings (Table 4.3).

The number-average molecular weight of each gingival sulphated glycosaminoglycan was thus determined by end group analysis. These were determined four times, for both 5 µl and 10 µl samples. The values, which ranged from 12,300 for heparan sulphate from the epithelium, to 27,000 for dermatan sulphate also from the epithelium, are listed in Table 4.3. A similar pattern of molecular weight distribution was noted for the connective tissue sulphated glycosaminoglycans, with heparan sulphate being the smallest (15,900) and dermatan sulphate the largest (25,000). Furthermore, there appeared to be only slight variations in the molecular weights of similar glycosaminoglycan species from epithelium and connective tissue.

DISCUSSION

In order to study the molecular weight of any specific tissue bound component, it is essential that it be extracted with minimal degradation. Although papain digestion of tissues is a common means of cleaving glycosaminoglycans from proteoglycans, incomplete degradation

Table 4.3

Number-Average Molecular Weight Estimations

GAG	Weight of GAG (μg)				Radioactivity (d.p.m.)				10^{-4} x Number-Average Molecular Weight	
	Epithelium		Connective Tissue		Epithelium		Connective Tissue		Epithelium	Connective Tissue
	5μl	10μl	5μl	10μl	5μl	10μl	5μl	10μl		
HS	2.9(± .09)	6.5(± .23)	2.5(± .07)	4.3(± .21)	145(± 2.6)	320(± 10.7)	95(± 2.6)	162(± 7.6)	1.23(± .04)	1.59(± .06)
DS	1.5(± .05)	2.9(± .09)	11.3(± .41)	21.4(± .7)	35(± .9)	70(± 1.7)	270(±11.2)	532(±31.1)	2.7 (± .08)	2.5 (± .13)
ChS4	1.9(± .09)	3.5(±.11)	4.5(± .13)	9.3(±.33)	45(± 1.7)	85(± 3.9)	130(± 4.2)	260(± 8.7)	2.5 (± .08)	2.1 (± .06)

The amounts (weight) of glycosaminoglycan separated from gingival epithelium and underlying connective tissue were determined by densitometric scans of the electrophoretograms and are expressed in μg for both 5 μl and 10 μl loadings. The level of radioactivity, expressed as d.p.m., is shown for isolated glycosaminoglycan species cut from the strips for both 5 μl and 10 μl loadings. The number-average molecular weights of the separated sulphated glycosaminoglycans were calculated from the recorded weights of the glycosaminoglycans and the specific activity of the NaB^3H_4 (6.03×10^5 d.p.m./μM) by reference to the d.p.m. of each electrophoretically separated band. All mean values were determined together with the standard error of the mean (in brackets) from four separate readings except for the molecular weight values which are the mean values obtained from both the 5 μl and 10 μl data. Abbreviations: GAG (glycosaminoglycan); HS (heparan sulphate) DS (dermatan sulphate); ChS4 (chondroitin sulphate 4).

of proteoglycan may yield single polysaccharide chains containing peptide contaminants together with random cleavage of glycosidic bonds within the polysaccharide chain (GREGORY and RODÉN, 1961; HOFFMAN, MASHBURN and MEYER, 1967; LUSCOMBE and PHELPS, 1967 a). Therefore, the digestion of gingival tissue with papain to obtain glycosaminoglycans for molecular weight analysis is not appropriate.

Following the demonstration of the cleavage under alkali conditions of a xylose-serine bond between glycosaminoglycans and proteoglycans (MUIR, 1958) and the subsequent confirmation that this represented a β-elimination reaction (ANDERSON, HOFFMAN and MEYER, 1965), investigators have established that most mammalian glycosaminoglycans (with the possible exception of hyaluronic acid) are covalently bound to a protein core via O-glycosidic bonds which are themselves alkali labile (ANDERSON, HOFFMAN and MEYER, 1965; BELLA and DANISHEFSKY, 1968; STERN, et al., 1969). Furthermore, since non-specific cleavage along the glycosaminoglycan chain does not occur following alkali treatment of proteoglycans (ROBINSON and HOPWOOD, 1973), alkali extraction represents a satisfactory means of obtaining glycosaminoglycans from tissue for structural and molecular weight analyses.

Alkaline treatment of human gingival epithelium resulted in extraction of glycosaminoglycans which represented 0.08% of the dry weight of the tissue. One hundred per cent extraction was assumed since there was no residual tissue left. This compares favourably with a figure of 0.07% obtained following papain digestion of human gingival epithelium (Chapter 3). On the other hand, only between 85-90% of the total tissue glycosaminoglycans were extracted, under alkaline conditions, from human gingival connective tissue. The

remaining 10-15% were released only after subsequent digestion with papain. The differences between alkali extractability of glycosaminoglycan species from gingival epithelium and connective tissue are unlikely to represent differences in modes of attachment of some glycosaminoglycan chains to protein between these two tissue types. Rather, they are more likely to reflect differences in the overall molecular architecture and size of the tissue specimens. The gingival connective tissue pieces were much larger (approximately 5 mm x 2 mm x 2 mm) and contain proportionately larger amounts of dense fibrous protein (e.g. collagen) which are no doubt more resistant to the action of chemicals and enzymes. In contrast, the epithelial specimens, although of the same length and breadth, are considerably thinner and more cellular and would be more susceptible to disaggregative processes.

Following confirmation that the methods of reductive end labelling, electrophoresis and densitometric quantitation were satisfactory for estimating molecular weights of standard glycosaminoglycan samples, various attempts were made to improve the specificity of electrophoretic separation of gingival glycosaminoglycans. In the previous chapter the separation of hyaluronic acid and heparan sulphate had not always been well resolved, and, whilst it was possible to identify each band by densitometry, visual assessment was sometimes difficult. Therefore, since it was neccesary to excise each Alcian Blue positive band to determine the amount of radioactivity present, a more distinct separation of hyaluronic acid and heparan sulphate was desirable.

Rather than rely upon complex discontinuous electrophoretic systems, the addition of 17% ethanol (v/v) to the electrophoretic

buffer (0.2 M calcium acetate pH 7.2) proved to be a satisfactory modification of the system previously used. Under these altered conditions, all glycosaminoglycans (standards and gingival) demonstrated slower elctrophoretic mobilities. The migration rate of heparan sulphate was most affected by the addition of ethanol to the electrophoretic buffer with gingival heparan sulphate demonstrating a slower migration than gingival hyaluronic acid, whilst the standard heparan sulphate sample appeared to move at approximately the same rate as standard hyaluronic acid.

Since the electrophoretic mobilities of glycosaminoglycans in calcium acetate buffer (without ethanol) are mainly dependent upon differences in the backbone structure of these polysaccharides as well as the positions of their sulphate groups (SENO, ANNO and KONDO, 1970), a possible explanation for the altered migration rates would be the association of ethanol with the glycosaminoglycans resulting in either structural or conformational changes leading to slower mobilities. On the other hand, the differential solubility of various glycosaminoglycans in aqueous ethanol, depending upon their state of hydration, may be responsible for altered electrophoretic mobilities. Furthermore, the addition of ethanol to the electrophoretic buffer may effectively reduce the number of free ions available for interaction with glycosaminoglycans by partially dehydrating the system and thereby affecting glycosaminoglycan mobility.

The difference in mobility of gingival and standard heparan sulphate relative to hyaluronic acid is more difficult to account for. However, the most likely explanation for these observations is that gingival heparan sulphate differs sufficiently in its backbone composition or degree of sulphation from the standard heparan sulphate employed (which was derived from beef lung) to result in a different electrophoretic

migration rate. Indeed, this would seem likely on the basis of the heterogenous nature of the heparan sulphates as a class of macromolecules (Chapter 1), and is certainly an area which could be further investigated.

The number-average molecular weight estimation of the sulphated glycosaminoglycans in human gingival epithelium and connective tissue revealed that in both tissues, heparan sulphate had the smallest value whilst dermatan sulphate and chondroitin sulphate possessed larger values which were similar. A value for hyaluronic acid has not been included in these results because the effect of alkali along its chain length is not yet clear. Indeed, it appears that some non-specific degradation of the hyaluronic acid molecule occurs in the presence of alkali (see Chapter 5).

Of teleological interest from this study is the additional information which describes the chemical composition of the extracellular matrix of human gingival epithelium and connective tissue; in particular is the confirmation using a different extraction procedure to that previously described in Chapter 3 that hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 are the constituent glycosaminoglycans in these tissues.

Furthermore, the molecular weight values obtained for each sulphated glycosaminoglycan indicated a range from 15,000 for the smallest (epithelial heparan sulphate) to 27,000 for the largest (epithelial dermatan sulphate). There was very little difference noted in the molecular weights of similar glycosaminoglycan species extracted from epithelium and connective tissue. The actual size of these molecules is not particularly noteworthy for connective tissues but when one considers the limited amount of "extracellular space" present in epithelium, one may speculate that molecules of this size

would assume highly complex conformations which in themselves would regulate inter-molecular interactions.

SUMMARY

The number-average molecular weights of microgram quantities of human gingival epithelial and connective tissue sulphated glycosaminoglycans have been determined.

Radioactive labelled glycosaminoglycans were extracted from separated human gingival epithelium and connective tissue following alkaline degradation of the tissues in the presence of tritiated sodium borohydride. These were identified electrophoretically as heparan sulphate, hyaluronic acid, dermatan sulphate and chondroitin sulphate 4. Following densitometric quantitation of the sulphated glycosaminoglycans (heparan sulphate, dermatan sulphate and chondroitin sulphate 4), the amount of radioactivity associated with each species was determined by liquid scintillation counting of each band staining positively with Alcian Blue for these glycosaminoglycans.

The number-average molecular weights for each glycosaminoglycan were thus determined by end group analysis. The molecular weight values obtained for each sulphated glycosaminoglycan indicated a range from 15,000 for epithelial heparan sulphate to 27,000 for dermatan sulphate also from the epithelium. There were little inter-tissue differences in molecular weight between similar species of sulphated glycosaminoglycans.

CHAPTER 5

MOLECULAR WEIGHT ESTIMATION OF GINGIVAL HYALURONIC ACID

INTRODUCTION

Currently, various methods are cited for determination of the molecular weight of hyaluronic acid. They include the use of sedimentation and diffusion data obtained with the ultracentrifuge (VARGA, 1955; SWANN, 1968 a & b), viscometry (LAURENT, 1955), light scattering (PRESTON, DAVIES and OGSTON, 1965; MEYER, PRESTON and LOWTHER, 1969) and streaming birefringence (ROWEN, BRUNISH and BISHOP, 1956). The wide distribution of molecular weights which have been reported for preparations of hyaluronic acid extracted from a variety of sources range from 10^4 to 10^6 (LAURENT, RYAN and PIETRUSZKIEWICZ, 1960; SWANN, 1969 a & b; SHIMADA and MATSUMARA, 1975). Indeed, hyaluronic acid has, by far, the largest molecular weight of the seven known mammalian glycosaminoglycans. Nonetheless, such reported polydispersity of molecular weight together with a tendency for hyaluronic acid to depolymerize during the different methods of preparation, as well as the variety of physicochemical techniques used are probably responsible for the above disparities.

Hyaluronic acid has previously been shown to be present in human gingiva as representing 5.2% and 3.6% of the total glycosaminoglycans of epithelium and connective tissue respectively (Chapter 3).

The molecular weights of gingival sulphated glycosaminoglycan components have been estimated by end group analysis following alkaline extraction in the presence of tritiated sodium borohydride (Chapter 4). However, the molecular weight of the hyaluronic acid component of these closely apposed tissues has not been established since such chemical analysis resulted in uncontrolled degradation of the hyaluronic acid molecules. As a consequence, this chapter describes the estimation of the molecular weight of hyaluronic acid isolated from both human gingival epithelium and connective tissue by

sedimentation measurements obtained by analytical ultracentrifugation.

MATERIALS & METHODS

Preparation of Gingival Hyaluronic Acid

In order to obtain hyaluronic acid from gingival tissue in a state resembling as close as possible that found in an *in vivo* situation, neither papain digestion nor alkaline hydrolysis was used. Papain digestion was considered unsuitable because of possible hyaluronic acid depolymerization by oxidation-reduction reactions due to the presence of cysteine in the buffer. On the other hand, alkaline hydrolysis appeared to result in uncontrolled degradation of gingival hyaluronic acid resulting in the extraction of a very small molecular weight material (see results this chapter).

After pooling the separated gingival epithelium and connective tissue in acetone, the dry defatted specimens (approximately 3.0 to 4.0 gm dry weight of tissue) were digested by pronase B (Calbiochem Behring Corp., La Jolla, California) to release the glycosaminoglycans. This digestion was performed in 0.5 M sodium acetate buffer, pH 7.5 (50 mg tissue/ml buffer) at 65^{o}C for 24 hours (0.4% pronase in 0.5 M sodium acetate buffer was added in two separate doses at 12 hour intervals to provide a final total of 10 µg enzyme/mg dry tissue).

Following digestion, protein was removed by adding 80% trichloroacetic acid to give a final concentration of 10% and further washed with 5% trichloroacetic acid. Pooled supernatants were dialysed against deionized water for 3 days at 4^{o}C. The retentate was then concentrated to a known small volume by rotary evaporation and the glycosaminoglycans precipitated by the addition of 4 volumes of 1% sodium acetate in absolute ethanol.

The precipitated glycosaminoglycans were recovered by centrifugation and subsequently dried *in vacuo* over phosphorous pentoxide.

Fractionation of the hyaluronic acid from the other sulphated glycosaminoglycans was carried out essentially as described by SCOTT (1960) in the presence of cetylpyridinium chloride (CPC). The sulphated glycosaminoglycans were precipitated in 0.15 M Na_2SO_4, containing 0.1% CPC leaving hyaluronic acid in the supernatant. Following centrifugation to remove the precipitated sulphated glycosaminoglycans, the hyaluronic acid in the supernatant was precipitated by the addition of an equal volume of water to a concentration of 60 mM Na_2SO_4 and was recovered by centrifugation (1000g).

The CPC complexes were converted to sodium salts by precipitation with ethanolic sodium acetate following solubilization of the CPC complexes with 66% propan-1-ol. The previously precipitated sulphated glycosaminoglycans in the supernatant were also recovered by ethanol precipitation following solubilization in 66% propan-1-ol.

Confirmation of fractionation was achieved by electrophoresis on Cellogel (Chemetron, Milan, Italy) in 0.2 M calcium acetate, pH 7.2, as described previously (see Chapter 3, Materials and Methods).

Concentration Determination

The concentration of glucuronic acid in the hyaluronic acid preparation was determined by the method of BLUMENKRANTZ and ASBOE-HANSEN (1973) as described in Chapter 2, using D-glucuronic acid as the standard.

The relationship between weight of standard hyaluronic acid and glucuronic acid had previously been determined (APPENDIX 7). This calibration indicated a multiplication of 2.5 was required to convert the concentration of D-glucuronic acid to hyaluronic acid. At the lower concentrations of hyaluronic acid the Schlieren pattern peak heights were used to confirm the concentration. A standard curve of peak height against hyaluronic acid concentration is shown

in Figure 5.1.

Ultracentrifugation

Sedimentation velocity studies on extracted hyaluronic acid from epithelium and connective tissue were carried out on a Beckman Model E analytical ultracentrifuge. Samples of both standard hyaluronic acid and gingival hyaluronic acids were dissolved in a buffer containing 0.2 M NaCl, 7.7 mM NaH_2PO_4 and 2.3 mM KH_2PO_4, pH 7.3 and dialysed for 24 hours against this buffer at 4^oC. Subsequent dilutions using the dialysate were made to give concentrations ranging from 2.3 mg/ml to 0.15 mg/ml (of uronic acid). Analytical ultracentrifugation was carried out at 59,780 rpm in 12 mm double sector cells at 15^oC. The moving boundary was observed using Schlieren optics and photographically recorded at either 2 or 8 minute intervals maintaining the phase angle constant throughout. Measurements were made at 2.5 x magnification on a Nikon microcomparator by two observers and averaged readings were used.

Molecular Weight Estimations

The sedimentation coefficients (s) were calculated for various concentrations of hyaluronic acid preparations as per equation 1.

$$s = \frac{(\text{slope})\ (2.303/60)}{\left[(2\pi)\ \frac{(59,780)}{60}\right]^2} \qquad (1)$$

Where (slope) is the slope of the plots of distance moved by the boundary in the Schlieren pattern versus time for each hyaluronic acid concentration (distance per minute). The factor (2.303/60)

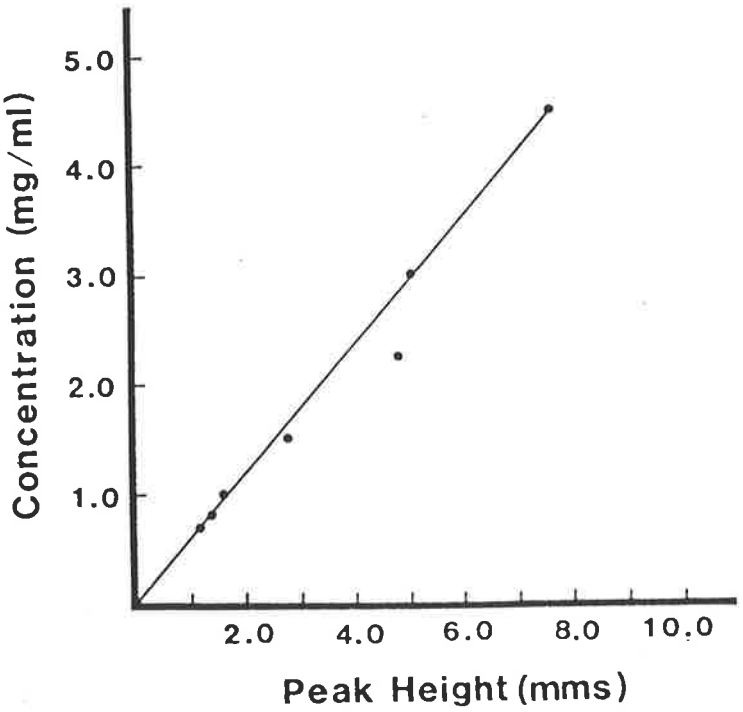


Figure 5.1

Relationship between Schlieren pattern peak height and hyaluronic acid concentration.

converts log 10 to natural logs and minutes to seconds. The denominator converts revolutions per minute (59,780) to radians per second.

The calculated values for s were converted to reciprocals and plotted as a range of values against concentration of hyaluronic acid. The values for $(s^{-1})_o$ (the reciprocal of the sedimentation coefficient at zero concentration) were obtained by extrapolation of a line of best fit to zero concentration. Both linear and parabolic regression functions were used (see later section, page 122).

Values for $(s^{-1})_o$, obtained for both standard samples of hyaluronic acid and gingival hyaluronic acid, were substituted into a calibration procedure previously determined by CLELAND and WANG (1970). In their procedure, data from hyaluronic acids of varying molecular weights were analysed in 0.2 M NaCl.

The equation:

$$\log (s^{-1})_o = 14.681 - 0.413 \log M_{sD} \qquad (2)$$

was applicable for hyaluronic acids with molecular weights above 10^5, where $(s^{-1})_o$ = reciprocal of the sedimentation coefficient at zero concentration and M_{sD} = molecular weight. The standard hyaluronic acid was used to confirm the substitution of our data into the above equation. Due to the limited availability of material the molecular weights of hyaluronic acid from gingival sources were obtained without resorting to viscometric or diffusion studies.

Statistical Methods

Extrapolation of the sedimentation data, s^{-1}, to values for $(s^{-1})_0$ at concentration = 0 was determined by fitting straight line regression (equation 3) and curvilinear regression (equqtion 4) curves to the data obtained; the standard error of the estimate of y was also determined (equation 5).

$$y = a + bx \quad (3)$$

$$y = a + bx + cx^2 \quad (4)$$

Where y represents y axis values (s^{-1}) and x represents x axis values (concentration) and a, b and c are constants.

$$S_{\hat{y}} = \sqrt{S^2_{yx}\left[\frac{1}{n} + \frac{(x_i - \bar{x})^2}{\Sigma(x - \bar{x})^2}\right]} \quad (5)$$

Where $S_{\hat{y}}$ = standard error of y; S^2_{yx} = standard deviation of the estimate; x_i = observed values; $\bar{x}$ = mean of observed values; n = number of observations.

RESULTS

Reductive End-labelling of Hyaluronic Acid

Values for the molecular weight of both standard and gingival hyaluronic acid were estimated by end group analysis following reductive end-labelling in the presence of alkaline tritiated sodium borohydride. In the first instance, standard hyaluronic acid was treated in a similar fashion as standard dermatan sulphate and chondroitin sulphate 4 as described in Chapter 4. The value thus derived for standard hyaluronic acid was 18,000 which compared with a reported value of 230,000 (see APPENDIX 6). This finding indicated that the above method may not be suitable for hyaluronic acid molecular weight estimation.

Similarly, hyaluronic acid extracted from human gingival epithelium and connective tissue under alkaline conditions in the presence of tritiated sodium borohydride indicated molecular weights of 52,000 and 26,000 respectively using the same method of calculation as described in Chapter 4. On the basis that hyaluronic acid from most tissues appears to range in molecular weight from 10^4 to 10^7 these values were considered as being too low.

Consequently, hyaluronic acid molecular weights were studied using another method, namely, analytical ultracentrifugation.

Fractionation of Gingival Hyaluronic Acid

Following pronase digestion of separated human gingival epithelium and connective tissue, hyaluronic acid was recovered by sequential precipitation in CPC and ethanol. The isolated hyaluronic acid was subsequently calculated to contribute 0.0053% and 0.0058% respectively of the dry weights of the tissues.

Confirmation of the isolation was demonstrated by electrophoresis and digestion of the hyaluronic acid isolates with Streptomyces hyaluronidase (Figure 5.2). Figure 5.2.a. shows an electrophoretogram of a mixture of standard glycosaminoglycans (hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4). Figure 5.2.b. reveals a single Alcian Blue positive band which could be removed by Streptomyces hyaluronidase and corresponded to hyaluronic acid. Figure 5.2.c. shows bands which represent the sulphated glycosaminoglycans of human gingivae (heparan sulphate, dermatan sulphate and chondroitin sulphate 4).

Purity of Hyaluronic Acid Preparation

Despite complete proteinase digestion of gingival epithelium and connective tissue with pronase, the possibility of some residual protein in the hyaluronic acid preparations could not be discounted. Therefore, all hyaluronic acid samples prepared from human gingivae were also assayed for protein. In all cases, the range of protein contribution to these preparations was not greater than 2% of the total weight of freeze dried hyaluronic acid. Differences in protein content of epithelial and connective tissue hyaluronic acid were noted.

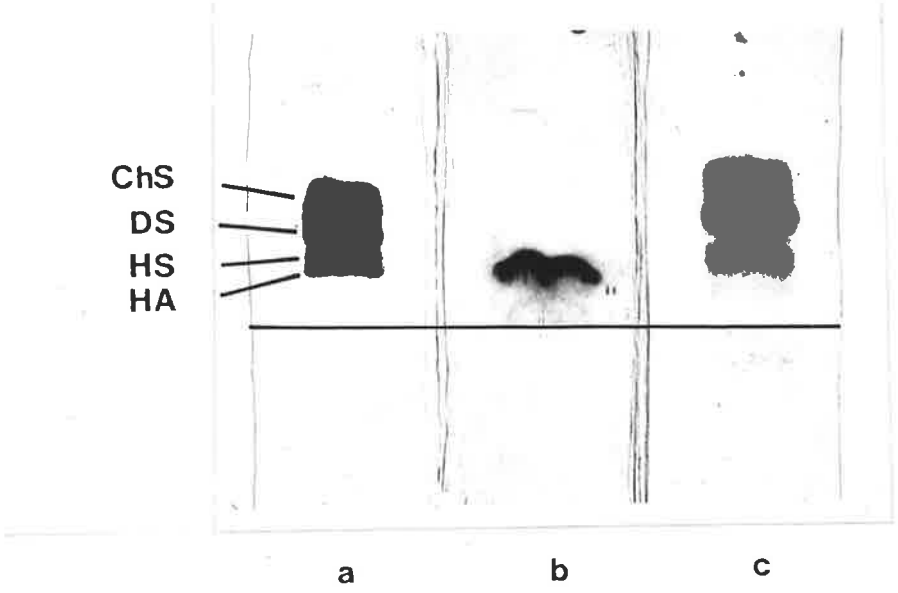


Figure 5.2

Electrophoretograms of glycosaminoglycans in 0.2 M calcium acetate, pH 7.2 at 30 volts/cm for 30 minutes.

a) Standard glycosaminoglycans (hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4).

b) Hyaluronic acid isolated from human gingivae.

c) Sulphated glycosaminoglycans extracted from human gingivae.

Abbreviations: HA (hyaluronic acid); HS (heparan sulphate); DS (dermatan sulphate); ChS (chondroitin sulphate 4).

For example, the greatest difference observed was in a preparation in which the connective tissue hyaluronic acid-protein content was 1.24% whilst its corresponding epithelial preparation was 0.6% of the hyaluronic acid dry weight. In all cases the protein contribution to hyaluronic acid was greatest for the connective tissue.

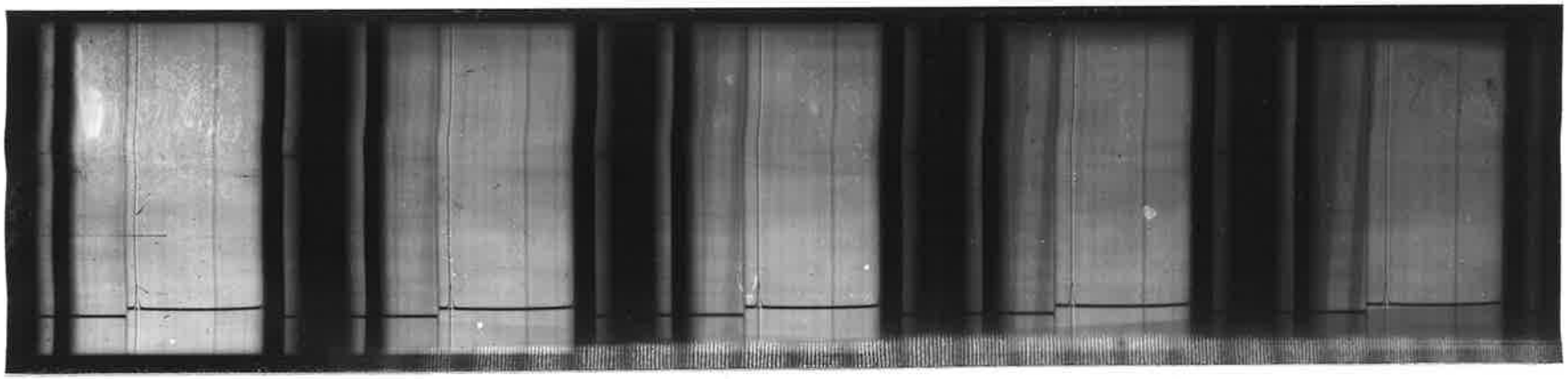
Sedimentation Velocity Studies

Figure 5.3 depicts the sedimentation velocity Schlieren patterns of hyaluronic acid fractionated from human gingival epithelium and connective tissue. Both samples produced hypersharp peaks in the ultracentrifuge, characteristic of homogeneous hyaluronic acid

Values for $(s^{-1})_o$ for epithelial, connective tissue and standard hyaluronic acid preparations were obtained by plotting s^{-1} against hyaluronic acid concentration (Figure 5.4) and are listed in Table 5.1. These values were derived by fitting the experimental data to either a linear or curvilinear regression analysis (equations 3 and 4). Included in Figure 5.4 are values derived from subjecting other published data (LAURENT, RYAN and PIETRUSZKIEWICZ, 1969; CLELAND and WANG, 1970; MATHEWS, 1977) to similar mathematical procedures.

The plots of s^{-1} against concentration for both the epithelial hyaluronic acid and the published values for high molecular weight hyaluronic acid (LAURENT, RYAN and PIETRUSZKIEWCZ, 1960) were best described by a parabolic function for all observed points. Such a derivation implies that s^{-1} values for these macromolecules were more dependent upon concentration than molecular size. Nonetheless, straight line relationships could be obtained for the sedimentation data as the concentration of hyaluronic acid approached zero. Thus, linear regression for points at the lower concentrations have been

a



b

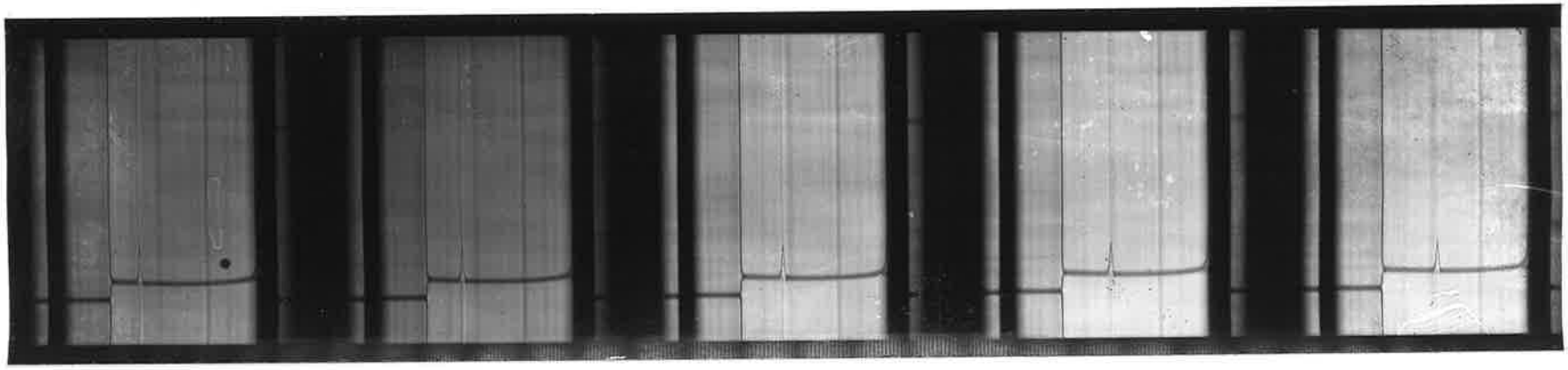


Figure 5.3

Sedimentation velocity Schlieren patterns of (a) gingival epithelial hyaluronic acid and (b) gingival connective tissue hyaluronic acid, concentration of approximately 2.5 mg/ml in 0.2 M NaCl in phosphate buffer pH 7.3. Sedimentation is from left to right. Runs were made at 59,780 rpm in 12 mm double sector cells. Photographs were taken at 8 minute intervals.

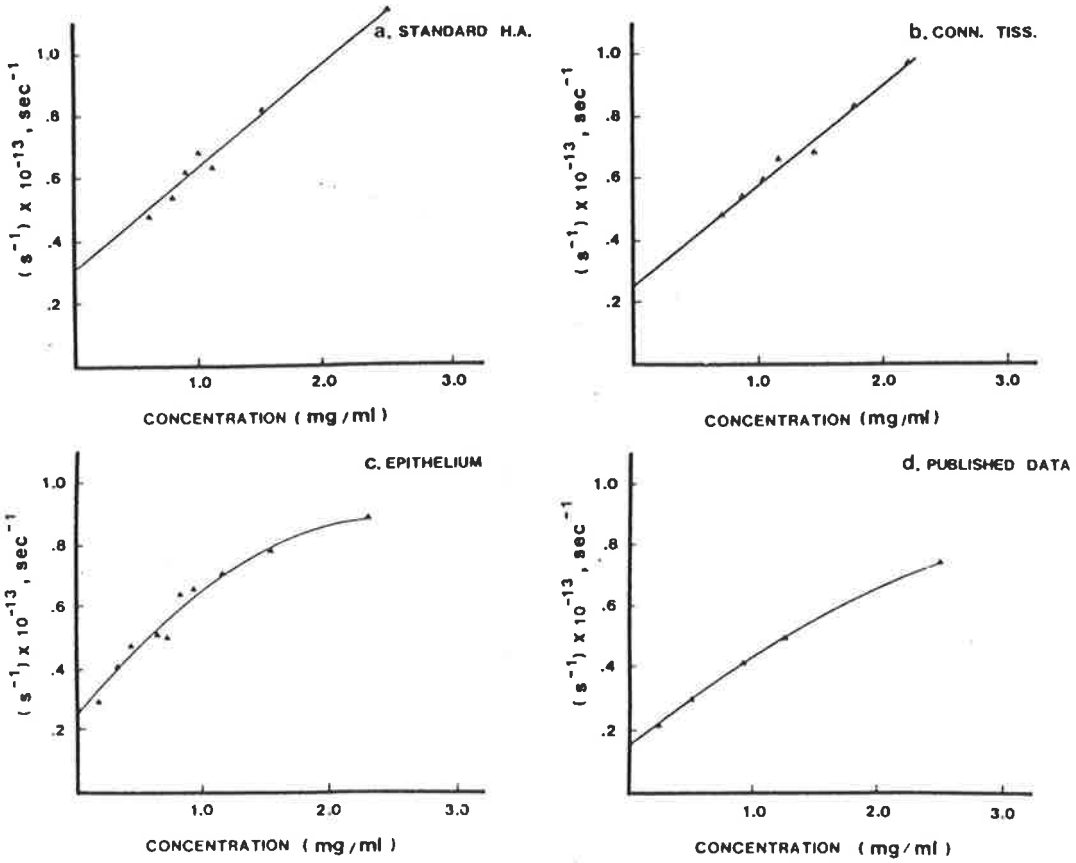


Figure 5.4

Reciprocal sedimentation velocity as a function of concentration.

a) Standard hyaluronic acid.

b) Connective tissue hyaluronic acid.

c) Epithelial hyaluronic acid.

d) Published data of LAURENT, RYAN and PIETRUSZKIEWCZ (1960).

Table 5.1

Reciprocal Sedimentation Values

Epithelium		Connective Tissue		Standard Hyaluronic Acid	
Conc. (mg/ml)	$(s^{-1})x10^{-13}$, sec^{-1}	Conc. (mg/ml)	$(s^{-1})x10^{-13}$, sec^{-1}	Conc. (mg/ml)	$(s^{-1})x10^{-13}$, sec^{-1}
2.3	0.88	2.2	0.97	2.5	1.14
1.53	0.77	1.76	0.83	1.5	0.82
1.15	0.69	1.46	0.68	1.125	0.63
0.92	0.65	1.2	0.66	1.0	0.68
0.766	0.64	1.1	0.60	0.9	0.63
0.7	0.49	0.88	0.54	0.8	0.53
0.66	0.50	0.73	0.48	0.6	0.48
0.4	0.47				
0.3	0.40				
0.15	0.28				

Reciprocal sedimentation values were obtained by calculating sedimentation coefficients at various concentrations using equation (1).

$$s = \frac{(\text{slope})\ (2.303/60)}{(2\pi)\ \left(\frac{59,780}{60}\right)^2}$$

calculated on the basis that the lowest concentrations analysed are the most reliable indicators of the position of $(s^{-1})_o$ since they are, per se, the closest to zero concentration.

On the other hand, when s^{-1} values of the standard hyaluronic acid of known molecular weight and of the connective tissue hyaluronic acid were plotted against concentration, linearity of all points was observed.

The s^{-1} values, standard errors of these estimates and molecular weight estimations for the epithelial, connective tissue and standard hyaluronic acid samples together with the values derived from the previously published data of LAURENT, RYAN and PIETRUSZKIEWICZ, (1960) are shown in Table 5.2.

On the basis of the regression analyses, standard hyaluronic acid revealed a derived M_{sD} of 205,000 which compares with a reported value of 230,000 at the time of original preparation (MATHEWS, 1977) (APPENDIX 6). The connective tissue hyaluronic acid $(s^{-1})_o$ intercept was also determined by linear regression of the s^{-1} values and indicated an M_{sD} of 340,000.

When the published data of LAURENT, RYAN and PIETRUSZKIEWICZ (1960) were subjected to curvilinear regression of all points, as well as to linear regression for their lowest observed concentrations (0.25, 0.5 and 0.9 mg/ml), M_{sD} values of 1.64×10^6 and 1.37×10^6 were determined respectively as compared with their reported M_{sD} value of 1.7×10^6.

On the basis of curvilinear regression analysis of all experimental points for epithelial hyaluronic acid, the M_{sD} value of 372,000 was obtained. A standard error of this estimate indicated a range of molecular weight values between 296,000 and 480,000. However, using the s^{-1} values for the three lowest concentrations of epithelial hyaluronic acid, a straight line relationship with

Table 5.2

Molecular Weight Estimations of Hyaluronic Acid by Two Regression Procedures

	STD. HYALURONIC ACID	CONNECTIVE TISSUE	EPITHELIUM		PUBLISHED DATA	
			Regression		Regression	
	Linear Regression	Linear Regression	Linear (lowest conc.)	Parabolic	Linear (lowest conc.)	Parabolic
$(s^{-1})_o x10^{-13}, sec^{-1}$	0.31	0.25	0.17	0.24	0.14	0.13
Standard Error of y estimate	0.033	0.028	0.01	0.024	0.007	0.01
Molecular Weight x 10^{-4} (estimated from y intercept)	20.5	33.7	86.0	37.2	137	164

The reciprocal sedimentation values of epithelial, connective tissue and standard hyaluronic acid have been determined by analytical ultracentrifugation. The previously published data of LAURENT, RYAN and PIETRUSZKIEWICZ (1960) were included to illustrate the effect of extrapolation of independent data to establish molecular weight. Linear regression curves were determined for concentrations less than 1.0 mg/ml for the epithelial data and published data, whilst the parabolic function included all experimentally derived data. The linear functions of standard and connective tissue hyaluronic acid were for all experimentally derived points.

a correlation coefficient of 0.99 and an $(s^{-1})_o$ intercept of 0.17 ($\pm$0.01) x 10^{13}, sec^{-1} which corresponded to an M_{sD} of 860,000 was obtained.

DISCUSSION

The proportionately small amounts of hyaluronic acid in gingival epithelium and connective tissue appear to be similar when compared with the tissue dry weights. However, the structural compositions of each of these two closely apposed tissues are quite distinct. Epithelium is a highly cellular tissue with minimal "extracellular space" whilst the markedly less cellular connective tissue is composed of fibres (collagen and elastin etc.) embedded in the gel of an extracellular matrix. The molecular sizes of hyaluronic acid in these two extracellular compartments are important if the interactive and biosynthetic roles which extracellular materials play in maintaining tissue integrity are to be understood. Previous work from our laboratories has implied a specific interaction between hyaluronic acid and epithelial proteoglycans, but not with the connective tissue proteoglycans (WIEBKIN, BARTOLD and THONARD, 1979). Furthermore, hyaluronic acid appears to be regulatory in the biosynthesis of proteoglycans in gingival epithelium (WIEBKIN and THONARD, 1982).

However, structural and biosynthetic studies on gingival hyaluronic acid are complicated by the extremely limited amount of material available. Indeed, the studies reported in this chapter highlight many of the problems associated with studying very small quantities of hyaluronic acid. That is, despite attempts to obtain as much gingival tissue for analysis as possible, and careful preparative procedures, only estimates based upon statistical limits have provided the molecular weights reported herein.

Nonetheless, at present, sedimentation values obtained from standard hyaluronic acid fractionated from other glycosaminoglycans, together with reference to an analysis of the previously published data of LAURENT, RYAN and PIETRUSZKIEWCZ (1960) have provided what appears to be a reliable basis for a statistically defined method of estimating the molecular weight of hyaluronic acid.

The use of the linear regression equation (equation 3) for analysis of the reciprocal sedimentation coefficients of connective tissue hyaluronic acid at various concentrations resulted in a derived $(s^{-1})_o$ value of 0.25 ($\pm$0.028) x 10^{13}, sec^{-1}. When these data were subjected to the equation proposed by CLELAND and WANG (1970), an M_{sD} value of 340,000 was obtained. This value was considered statistically reliable since the correlation coefficient of the line was 0.97 throughout the whole range of concentrations used. However, experimental data obtained for epithelial hyaluronic acid preparations demonstrated a concentration dependence in that the derivation of a curvilinear relationship was achieved (Figure 5.4).

Since the published data of LAURENT, RYAN and PIETRUSZKIEWICZ (1960) for high molecular weight hyaluronic acid indicated similar curvilinear relationships, the simple parabolic function (equation 4) was used for both LAURENT'S data and that obtained from gingival epithelial hyaluronic acid. Whilst LAURENT, RYAN and PIETRUSZKIEWICZ (1960) did not reveal what function they used to obtain an $(s^{-1})_o$ intercept which corresponded to an M_{sD} value of 1.7 x 10^6, the calculation of 1.6 x 10^6 using a parabolic extrapolation procedure approaches that value. Nevertheless, the use of a linear regression in the concentration independent portion of the curve is considered to provide a more reliable extrapolation to $(s^{-1})_o$. Values of s^{-1} for concentrations below those of LAURENT, RYAN and PIETRUSZKIEWICZ (1960) have been carefully

prepared for the epithelial hyaluronic acid and two observers measured the Schlieren patterns. The correlation coefficient of the three lowest concentrations was 0.99 and the intercept indicated an $(s^{-1})_o$ value of 0.17 which corresponds to an M_{sD} of 860,000.

Although the derived $(s^{-1})_o$ values from a curvilinear regression may be considered an adequate approximation for calculations of M_{sD} of non-ideal macromolecules, it is likely that the significance of an observed concentration dependent curve is simply to indicate the magnitude of the molecular weight and concomitant molecular entanglement during ultracentrifugation. Indeed, the present data would amplify this concept.

The M_{sD} values derived from a parabolic extrapolation of sedimentation data for epithelial hyaluronic acid indicate a molecular weight only 35,000 greater than that obtained by the linear regression of the connective tissue hyaluronic acid data. Such a small difference in calculated molecular weights does not adequately reflect the observable differences in sedimentation velocity-concentration plots. Therefore, a linear extrapolation of the s^{-1} values of epithelium hyaluronic acid was used in which the experimental values representing the line of best fit were selected on the basis that they were derived from those concentrations of hyaluronic acid which approached zero. Indeed, these findings corroborate those of CLELAND and WANG (1970) that the s^{-1} values of high molecular weight hyaluronic acids are not linear with concentration. Therefore, either a more elaborate extrapolation procedure or a very careful method of measurement of low concentrations of hyaluronic acid is required. In doing so, the effect of s^{-1} values at higher concentrations is minimized during the extrapolation procedures.

In addition, the present study also supports the earlier

observations of LAURENT, RYAN and PIETRUSZKIEWCZ (1960) that the sedimentation velocity of high molecular weight hyaluronic acid is independent of molecular weight at high concentrations, particularly above 2.0 mg/ml (as stated by these workers). Indeed, the current data would imply that the curvilinear nature of the plot should, in the first instance, only be used as an indication that the molecule is very large and consequently demonstrates non-ideality in the ultracentrifuge. Subsequent molecular weight estimations should, therefore, be made by extrapolation of the linear portion of the curve in the lower concentration ranges.

The contribution of the protein associated with the gingival hyaluronic acid preparations to behaviour in the analytical ultracentrifuge cannot be discounted. However, the sedimentation velocity differences noted are difficult to explain on the basis of protein composition, since the connective tissue hyaluronic acid contained more protein than the epithelial hyaluronic acid preparations. Whether the presence of protein alters the interactive capacity of hyaluronic acid during ultracentrifugation remains to be investigated. For the present study the presence of protein is acknowledged but no corrections were made for its variable contribution to the epithelial and connective tissue hyaluronic acid preparations.

The interpretation of the data presented in this study has therefore been regarded as approaching reasonable values for M_{sD} when considering the limited amount of material available. Indeed, this is the first report which has attempted to study hyaluronic acid molecular weights from "normal" human gingivae. Whilst the results presented in this study are based upon statistical extrapolation procedures, the method of hyaluronic acid molecular weight determination recently described by WIK (1979) and further developed by LAURENT (1982) could be adopted to confirm these findings.

In conclusion, the observation that the behaviour of hyaluronic acid from gingival epithelium and connective tissue in the analytical ultracentrifuge is different is considered to reflect differences in the molecular weights of hyaluronic acid isolated from the two tissues. This contrasts with the findings reported in Chapter 4, in which very little difference in the molecular weights of the sulphated glycosaminoglycans of gingival epithelium and connective tissue were noted. Whether these differences are relevant to variable functional capacities of hyaluronic acid within gingival epithelium and connective tissue remains to be investigated. Indeed, it is tempting to speculate that the differences in molecular weight of the two hyaluronic acid preparations reflect functional features of the molecules in the two tissues. Namely, that hyaluronic acid in epithelium is involved in proteoglycan aggregation whilst, in connective tissue, the absence of proteoglycans capable of forming aggregates mitigates against such hyaluronic acid functions (WIEBKIN, BARTOLD and THONARD, 1979).

SUMMARY

The molecular weights of hyaluronic acid isolated from separated specimens of gingival epithelium and connective tissue as well as a standard hyaluronic acid preparation have been estimated. The values were determined following substitution of sedimentation values obtained in an analytical ultracentrifuge into a previously determined relationship between the reciprocal of the sedimentation coefficient at zero concentration $(s^{-1})_o$ and molecular weights estimated by sedimentation-diffusion (M_{sD}). Values of $(s^{-1})_o$ for connective tissue and standard hyaluronic acid preparations were obtained by linear regression and indicated molecular weights

(M_{sD}) of 340,000 and 205,000 respectively. Epithelial hyaluronic acid behaved differently during ultracentrifugation and demonstrated a curvilinear relationship between s^{-1} and concentration. This relationship was linear only at the lower concentration range. Linear extrapolation of s^{-1} values at the lowest concentrations together with curvilinear extrapolation of all experimental data revealed a likely range of molecular weight (M_{sD}) for epithelial hyaluronic acid of 860,000 to 372,000. Similar treatment of s^{-1} values derived from the previously published data of LAURENT, RYAN and PIETRUSZKIEWICZ (1960) also demonstrated both curvilinear relationships for all points and linearity of s^{-1} at the lower concentrations, and indicated the above to be a statistically accurate means of estimating molecular weight when more complex techniques are not suitable due to the limited amounts of hyaluronic acid available for analysis.

CHAPTER 6

PROTEOGLYCANS OF HUMAN GINGIVAL EPITHELIUM AND CONNECTIVE TISSUE

INTRODUCTION

Despite knowledge that the proteoglycan molecule is the functional unit of the uronate containing macromolecules *in vivo*, the previous studies reported in this thesis have focused upon the glycosaminoglycan content alone. However, now that the types of glycosaminoglycans which constitute human gingival epithelium and connective tissue extracellular matrices have been identified, quantitated and partially characterized, it is appropriate to consider the proteoglycan molecules within these regions as integral units.

As mentioned earlier, there are very few published reports regarding gingival proteoglycans (see Chapter 1, Introduction). This is, indeed, surprising since it is the proteoglycans which can influence many important properties of connective tissues (COMPER and LAURENT, 1978). Consequently, alterations within the extracellular matrix, proteoglycans in particular, could be expected to have significant effects upon both the cellular components and the tissues which they comprise. Therefore, a tissue such as gingiva which is subjected to continual chemical and mechanical abuse would be expected to rely heavily upon its extracellular matrix to maintain tissue integrity as well as an environment satisfactory for adequate cellular activity.

This chapter reports the extraction of proteoglycans from separated specimens of human gingival epithelium and connective tissue and observations on their structural components and gel chromatographic behaviour.

MATERIALS AND METHODS

Extraction of Proteoglycans

Separated specimens of human gingival epithelium and connective tissue were pooled in acetone until enough material was available for analysis. In general, 1.0 to 2.0 gm of connective tissue and 2.0 to 3.0 gm of epithelium were the minimum dry weights of gingival tissue from which sufficient proteoglycans could be extracted for analysis.

The proteoglycans (1 gm tissue/15 ml buffer) were extracted in 4 M guanidinium chloride in 0.5 M sodium acetate pH 7.4, containing proteinase inhibitors as described by OEGEMA, HASCALL and DZIEWIATKOWSKI (1975). To this solution 1 mg/litre of Soya Bean Trypsin inhibitor (Sigma) and 0.1 ml Trasylol (1000 Kallikrein Inactivation Units, Bayer Pharmaceuticals, Sussex, U.K.) were added. The guanidinium chloride had previously been purified by stirring and filtering through activated charcoal. Extraction was carried out at $4^{o}C$ for 24 hours with gentle stirring. The suspension was filtered through glass wool and the residue re-extracted in fresh buffer for a further 24 hours at $4^{o}C$ with gentle stirring. The suspension was again filtered, and the residue washed with 5 ml of the guanidinium chloride buffer. The resultant extracts were pooled and concentrated to one-tenth the original volume in a Millipore ultrafiltration unit (Millipore Corporation, Massachusetts, U.S.A.) fitted with a Pellicon ultrafiltration membrane with a nominal molecular weight exclusion limit of 10,000 (Millipore Corporation, Massachusetts, U.S.A.).

Fractionation of Gingival Proteoglycans

The concentrated extract was dialysed at 4°C for 3 days against 7 M urea in 0.05 M Tris HCl, pH 6.5, made with urea from a stock solution previously deionized by filtration through a mixed anion-cation exchange resin. A small precipitate sometimes formed at this stage, however, as it appeared to contain no uronic acid it was discarded. The dialysed sample was then applied to a column (1 x 17 cm for connective tissue and 1 x 9 cm for epithelium) of Sephadex DEAE A-50 anion-exchange resin equilibrated with the same 7 M urea in 0.05 M Tris HCl buffer (ANTONOPOULOS, et al., 1974). Different sized ion-exchange columns were used because greater amounts of proteoglycans were extracted from the connective tissue than from the epithelium. The sample was added until uronic acid appeared in the column effluent, indicating saturation of the column. The bound material was eluted stepwise with (a) the starting buffer, (b) 0.15 M NaCl in the same buffer and, (c) 2.0 M NaCl in the same buffer. Aliquots of each fraction were measured for both uronic acid (BLUMENKRANTZ and ASBOE-HANSEN, 1973) and protein by direct absorbance at 280 nm.

The major uronic acid peak (2.0 M NaCl) contained most of the proteoglycans. A smaller peak was often seen with the 0.15 M NaCl elution but this contained predominantly hyaluronic acid. The 2.0 M NaCl fractions were pooled and dialysed against several changes of 0.05 M sodium acetate, pH 7.4 containing proteinase inhibitors at 4°C for 3 days and also dialysed against deionized water at 4°C for 1 day. In some cases a slight precipitate formed, which was separated from the rest of the retentate by centrifugation (1000g). This precipitate was insoluble in 4 M guanidinium chloride and partially soluble in concentrated sulphuric acid. Uronic acid analysis revealed the possibility of some uronic acid positive

material being present, but due to difficulties in reading the colour absorption at 520 nm as a result of interfering colours caused by the addition of sulphuric acid, quantitation of the uronic acid was not possible. No further analyses were performed on these precipitates due to their resistance to resolubilization. The retentate (free from precipitates) was then lyophilized.

Density Gradient Ultracentrifugation

A 4 M guanidinium chloride solution in 0.05 M sodium acetate, pH 7.4 containing proteinase inhibitors was adjusted to a starting density of 1.35 gm/ml by adding solid caesium chloride. The crude lyophilized proteoglycan preparation (3 mg/ml) was dissolved in this buffer. Centrifugation was performed in a Beckman Model L preparative ultracentrifuge fitted with a Type 40 rotor (12 x 10 ml) at 35,000 rpm at 15^{o}C for 48 hours. After centrifugation the tubes were rapidly frozen at -70^{o}C and cut into five fractions of equal volume. They were thus termed respectively D_E 1, D_E 2, D_E 3...etc. for the epithelium and D_{CT} 1, D_{CT} 2,...D_{CT} 5 for the connective tissue fractions; where fraction one was the most dense one-fifth. The densities of each fraction were subsequently measured and recorded using a 100 µl micropipette. Each fraction was then dialysed against deionized water to permit uronic acid (BLUMENKRANTZ and ASBOE-HANSEN, 1973) and protein (LOWRY, et al., 1951) measurements to be made free of interference from caesium chloride and guanidinium chloride.

Small aliquots of the dialysed fractions were taken for glycosaminoglycan analysis by electrophoresis.

Gel Chromatography

Fractionation of the purified proteoglycans was performed on Sepharose 4B-CL columns. Epithelial fractions (D_E 1,2 and 3 together) and connective tissue fractions (D_{CT} 1 and 2 separately) were applied onto columns of 29 cm x 0.6 cm and 61 cm x 0.9 cm respectively and eluted with 0.5 M sodium acetate, pH 7.4 or 4 M guanidinium chloride in 0.05 M sodium acetate, pH 7.4. Both buffers contained proteinase inhibitors.

In all cases constant flow rates were controlled by the use of a peristaltic pump. The void volume (V_o) was established by the elution of Blue Dextran 2000 (Pharmacia, South Seas, Sydney, Australia) whilst the total volume (V_t) was determined by the elution of a small sample of [^{35}S]-sulphate (Radiochemical Centre, Amersham, England).

Preparation and Electrophoresis of Glycosaminoglycans

The residual epithelium and connective tissue from the 4 M guanidinium chloride extraction was digested by papain E.C. 3.4.22.2. (B.D.H. Chemicals, England) to release any remaining unextracted glycosaminoglycans. The wet tissue (1 mg/ml buffer) was subjected to papain digestion and subsequent glycosaminoglycan recovery as described in Chapter 3.

Similarly, aliquots from each of the proteoglycan fractions obtained by density gradient ultracentrifugation and column chromatography on Sepharose 4B-CL were subjected to papain digestion and glycosaminoglycan recovery by ethanol precipitation (Chapter 3).

Electrophoresis of the recovered glycosaminoglycans was performed in 0.2 M calcium acetate, pH 7.2 on Cellogel cellulose acetate electrophoresis strips at 30 volts/cm for 30 minutes as previously described in Chapter 3. In some instances, heparan sulphate and hyaluronic

acid were superimposed. In these cases confirmation of molecular species was obtained by digestion with Streptomyces hyaluronidase as previously described (Chapter 3). Quantitation of the glycosaminoglycan species was achieved following integration of densitometric scans of electrophoresed specimens.

Amino Acid Analysis of Proteoglycan Core Protein

Samples of proteoglycan (0.2 mg) from the densest fractions only (i.e. D_E 1 and D_{CT} 1) of the caesium chloride density gradient fractions for both epithelium and connective tissue were hydrolysed in 6 M HCl for 24 hours. Subsequent amino acid analysis was carried out using a Beckman Model 120C amino acid analyser.

RESULTS

Extraction of Proteoglycans

Following the crude extraction of proteoglycans by guanidinium chloride and subsequent concentration by ultrafiltration, a preliminary proteoglycan preparation was obtained by ion-exchange chromatography on Sephadex DEAE A-50. This resulted in three peaks which contained both uronic acid and protein (Figure 6.1). The major uronic acid peak for both epithelium and connective tissue proteoglycan extracts was obtained following elution with 2.0 M NaCl. The smaller uronic acid peak observed in the 0.15 M NaCl eluent appeared to be hyaluronic acid (as judged by electrophoresis of the papain digest of this material) with no sulphated glycosaminoglycans being detected. The uronic acid peak seen with the initial 7.0 M urea elution of the columns was a consequence of overloading the ion-exchange resins. In all cases the columns were loaded with proteoglycan extract until

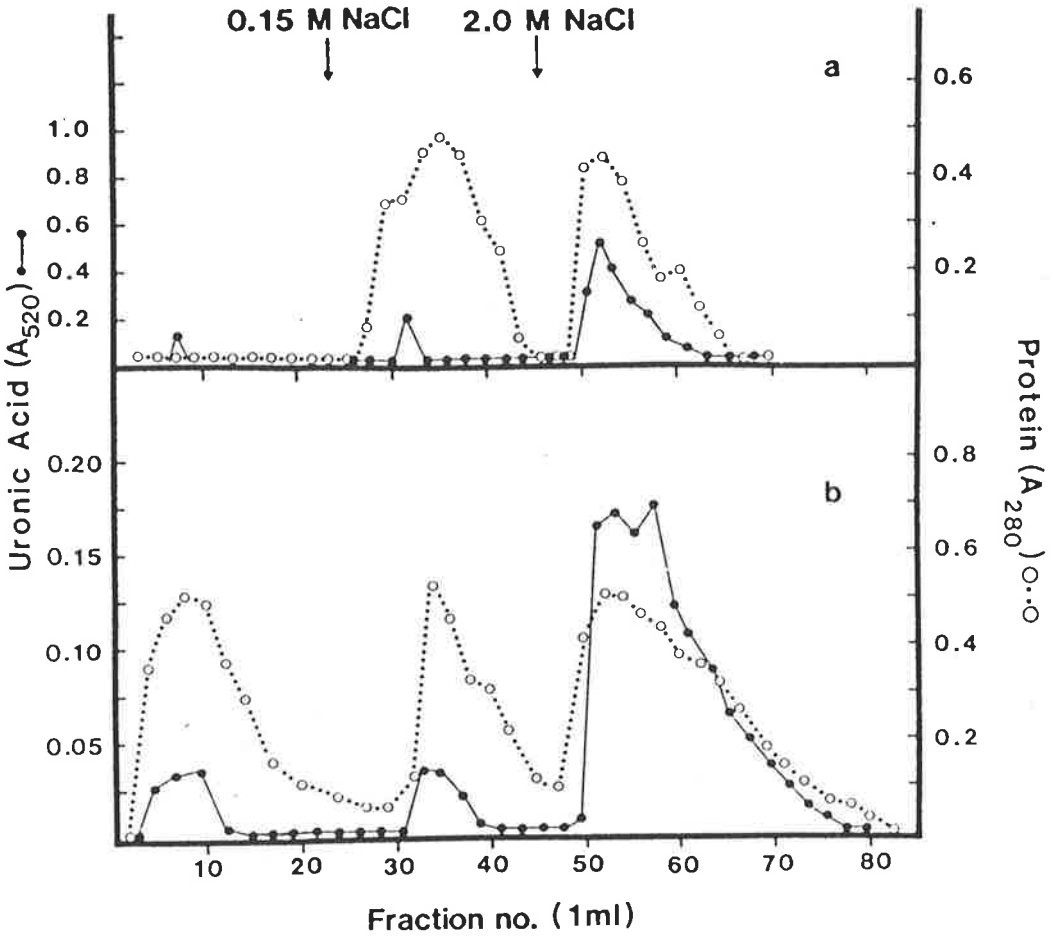


Figure 6.1

Ion-exchange chromatography of gingival proteoglycans. (a) Epithelial and (b) connective tissue proteoglycans extracted by 4 M guanidinium chloride in 0.05 M sodium acetate, pH 7.4, were applied to columns (1 cm x 9 cm for epithelium and 1 cm x 17 cm for connective tissue) of Sephadex DEAE A-50 equilibrated with 7 M urea in 0.05 M Tris HCl, pH 6.5. Elution was stepwise with (i) 7 M urea in 0.05 M Tris HCl; (ii) 0.15 M NaCl in same buffer and (iii) 2.0 M NaCl in same buffer. One ml fractions were collected. Flow rate was 4 ml/hour.

o··o protein (280 nm)

●—● uronic acid

uronic acid was detectable in the effluent. This was taken to indicate that the ion exchange resin was maximally bound with proteoglycan and stepwise elution could commence.

Papain digestion of the gingival tissue remaining after guanidinium chloride extraction, revealed that the 4 M guanidinium chloride treatment of gingival epithelium and connective tissue had extracted 95% and 85% respectively of the total epithelial and connective tissue uronic acid containing glycosaminoglycans (Table 6.1).

Fractionation of the proteoglycan rich fraction from ion-exchange chromatography (2.0 M NaCl peak) was achieved by density gradient ultracentrifugation in caesium chloride in 4 M guanidinium chloride with a starting density of 1.35 gm/ml (Figure 6.2 a and b). In these experiments, the epithelial proteoglycans appeared to be more evenly distributed throughout the denser three-fifths of the gradient (D_E 1,2 and 3) than the connective tissue proteoglycans which were mostly contained within the bottom two-fifths (D_{CT} 1 and 2). While some uronic acid was observed in the top two connective tissue fractions (D_{CT} 4 and 5), there was very little detectable uronic acid in the two least dense fractions of the epithelial extract. The protein profiles of material from both tissues were similar, with the greatest proportion of protein appearing in the least buoyant fractions.

Column Chromatography

Column chromatography of the epithelial and connective tissue proteoglycans prepared by density gradient centrifugation was performed on Sepharose 4B-CL eluted with 0.5 M sodium acetate, pH 7.4.

Table 6.1

Percentage of Proteoglycans Extracted from Gingival Epithelium and Connective Tissue by 4 M Guanidinium Chloride.

Effectiveness of Extraction Procedure	Epithelium	Connective Tissue
Extracted Proteoglycan (mg uronic acid)	1.28 (±0.39) mg	4.18 (±1.1) mg
Uronic acid released by subsequent papain digestion (mg uronic acid)	0.55 (±0.03) mg	1.14 (±0.15) mg
Percentage of proteoglycan extracted by 4 M guanidinium chloride	95%	79%

The proteoglycans were extracted by 4 M guanidinium chloride in 0.05 M sodium acetate, pH 7.4. The residual epithelial and connective tissue was digested by papain to release any unextracted glycosaminoglycans. Values are expressed as mg of uronic acid. Values in brackets are standard deviations.

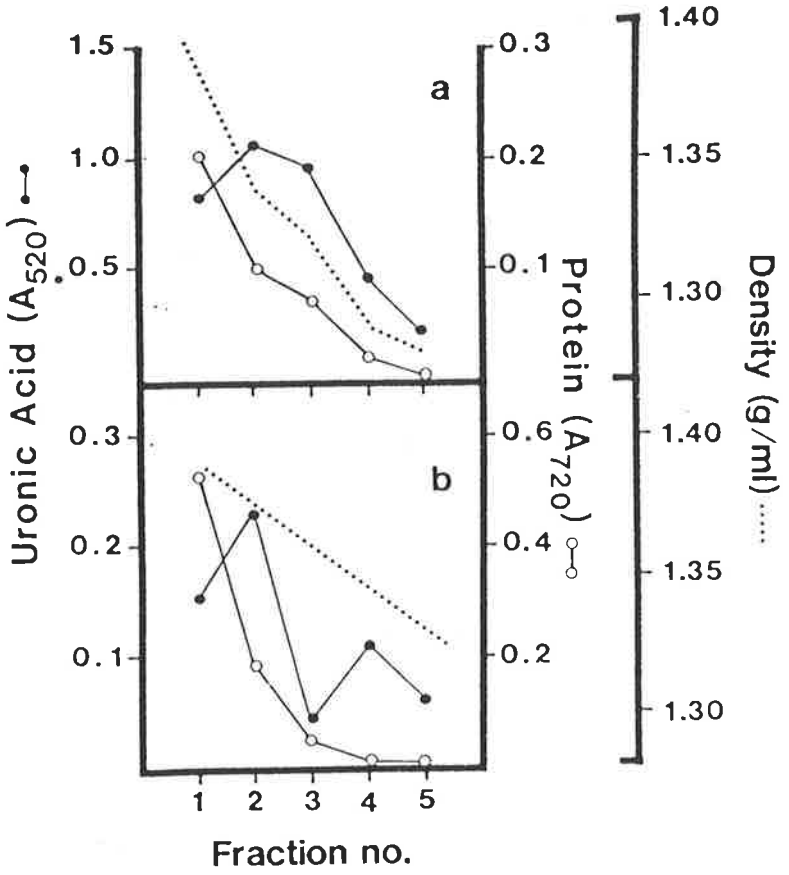


Figure 6.2

Density gradient centrifugation of gingival proteoglycans. (a) Epithelial and (b) connective tissue proteoglycan rich preparations obtained from Sephadex DEAE A-50 ion-exchange chromatography (2.0 M NaCl peaks) were dissolved in 4 M guanidinium chloride in 0.05 M sodium acetate adjusted to a starting density of 1.35 gm/ml with caesium chloride. Centrifugation was at 35,000 rpm for 48 hours. The fractions thus obtained were termed D_E 1; D_E 2; D_E 3; D_E 4; D_E 5 and D_{CT} 1; D_{CT} 2; D_{CT} 3; D_{CT} 4; D_{CT} 5 for epithelium and connective tissue respectively.

o—o protein (Folin)
●—● uronic acid
····· density

Attempts to fractionate the three densest epithelial density gradient fractions separately (D_E 1,2 and 3) were not successful due to the very limited amount of material available in each. Indeed, in many cases, no uronic acid profiles were obtained presumably because of dilution during chromatography. Therefore, epithelial proteoglycan fractions D_E 1,2 and 3 were pooled and then fractionated on Sepharose 4B-CL. This resulted in a single peak with a K_{av} of 0.53 (Figure 6.3). No uronic acid was detected at either the V_o or V_t of the column.

Since there were greater amounts of connective tissue proteoglycans recovered following density gradient centrifugation, the bottom two fractions (D_{CT} 1 and D_{CT} 2) could be fractionated separately on Sepharose 4B-CL with 0.5 M sodium acetate, pH 7.4. The proteoglycans of fraction D_{CT} 1 separated into two populations following such chromatography under associative conditions (Figure 6.4 a). One peak was excluded from the gel (K_{av} 0.08) whilst the other was included (K_{av} 0.46). Quantitatively, the major proportion of these two proteoglycans was comprised of the smaller included material. Chromatography of fraction D_{CT} 2 also resulted in two peaks of proteoglycans being eluted, both of which were included. The larger sized material eluted with a K_{av} of 0.31 whilst the smaller material appeared more polydisperse in size with an estimated K_{av} of 0.57 (Figure 6.4 b).

Since some of the proteoglycans of fraction D_{CT} 1 were excluded from Sepharose 4B-CL under associative conditions, this fraction was also chromatographed on Sepharose 4B-CL under dissociative conditions (4 M guanidinium chloride in 0.05 M sodium acetate, pH 7.4). A similar profile to that observed under associative conditions was observed (Figure 6.5). A small peak corresponding

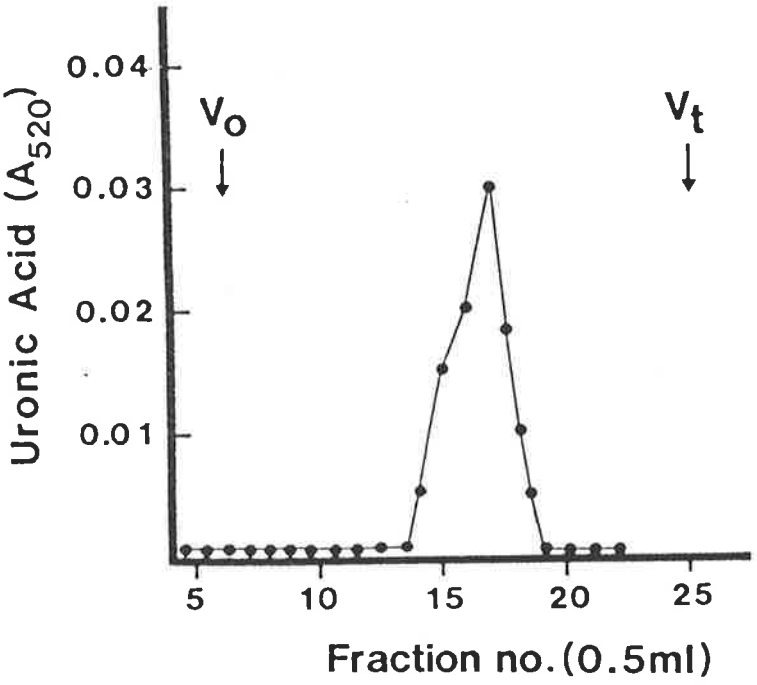


Figure 6.3

Gel chromatography of epithelial proteoglycans.

Proteoglycans from the pooled epithelial density gradient fractions D_E 1,2 and 3 were applied to a Sepharose 4B-CL column (0.6 cm x 29 cm) and eluted at 3 ml/hour under associative conditions (0.5 M sodium acetate, pH 7.4). Fractions of 0.5 ml were collected. V_o determined with Blue Dextran 2000 and V_t determined with [^{35}S]-sulphate.

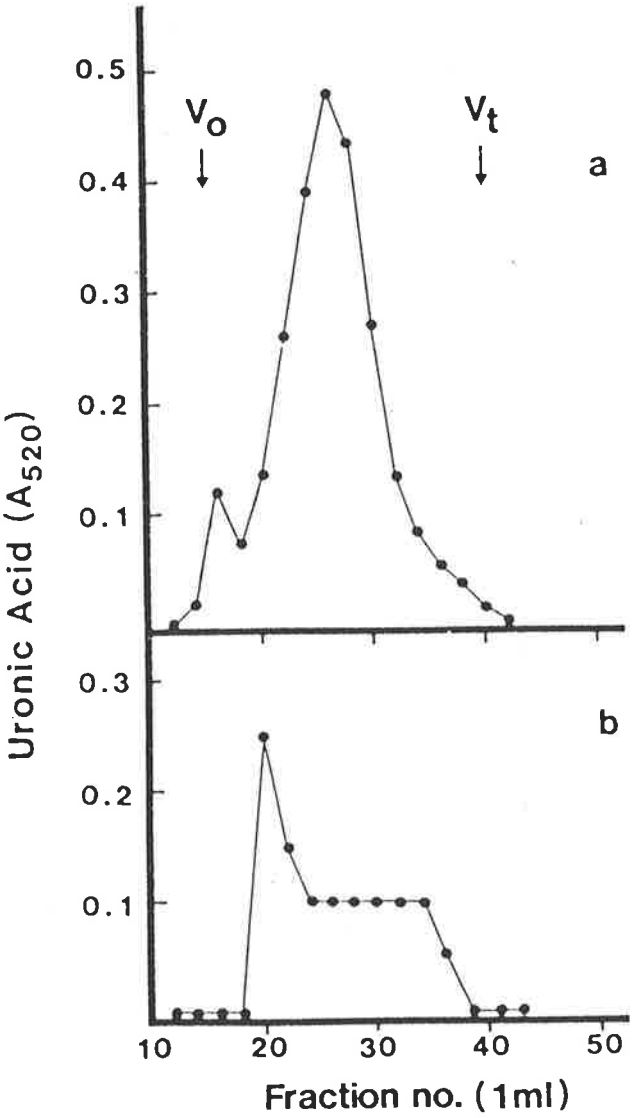


Figure 6.4

Gel chromatography of connective tissue proteoglycans under associative conditions. Proteoglycans from the connective tissue density gradient fractions (a) D_{CT} 1 and (b) D_{CT} 2 were applied separately to columns of Sepharose 4B-CL (0.9 cm x 61 cm) and eluted at 3 ml/hour under associative conditions (0.5 M sodium acetate, pH 7.4). Fractions of 1 ml were collected. V_o and V_t were determined as for Figure 6.3.

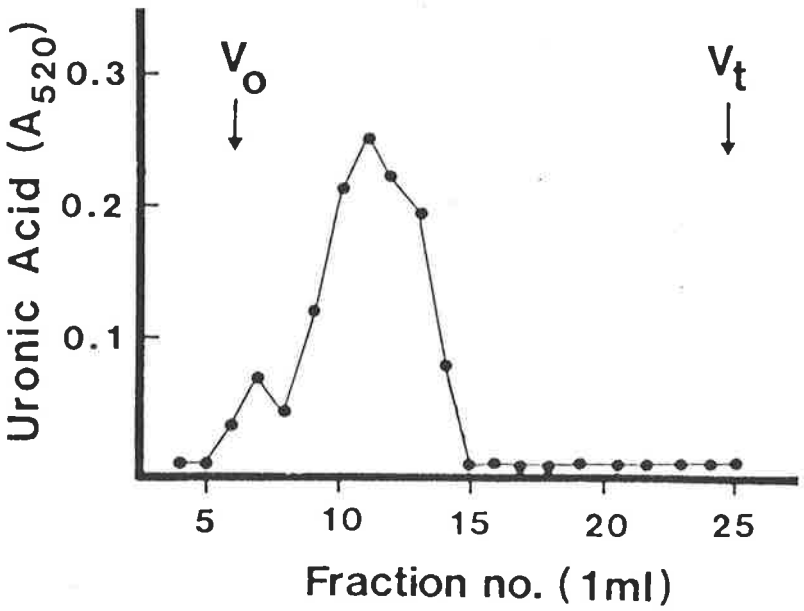


Figure 6.5

Gel chromatography of connective tissue proteoglycans under dissociative conditions. Proteoglycans from the D_{CT} 1 density gradient fraction were applied to a Sepharose 4B-CL column (0.6 cm x 29 cm) and eluted at 3 ml/hour under dissociative conditions (4 M guanidinium chloride in 0.05 M sodium acetate, pH 7.4). Fractions of 1.0 ml were collected. V_o and V_t were determined as for Figure 6.3.

to larger sized material was excluded from the gel (K_{av} 0.14) and the bulk of the proteoglycans included within the gel (K_{av} 0.57).

Glycosaminoglycan Analysis

Papain digestion of the residual gingival epithelium and connective tissue following guanidinium chloride extraction released the remaining glycosaminoglycans which could be identified by electrophoresis (Figure 6.6). In all cases, hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate 4 were released.

The proportions of each of the unextracted glycosaminoglycans in the epithelium and connective tissue are listed in Table 6.2. The major glycosaminoglycan which remained in the epithelial tissue was heparan sulphate (60%) whilst the major glycosaminoglycan not extracted by guanidinium chloride from the connective tissue was hyaluronic acid (30%).

Electrophoretic analysis of the constituent glycosaminolycans of gingival epithelial and connective tissue proteoglycans chromatographed on Sepharose 4B-CL can be seen in Figure 6.7. The relative amounts of each glycosaminoglycan in these proteoglycans are listed in Table 6.3. The epithelial proteoglycans appeared to be comprised of heparan sulphate and dermatan sulphate. No chondroitin sulphate or hyaluronic acid was detected. On the other hand, the glycosaminoglycans of the connective tissue proteoglycans corresponded to heparan sulphate, dermatan sulphate and chondroitin sulphate 4. No hyaluronic acid was detected (Figure 6.7).

Quantitatively, heparan sulphate was the major glycosaminoglycan of the epithelial proteoglycans (80%) whilst dermatan sulphate was the major glycosaminoglycan of the connective tissue proteoglycans (50.6%).

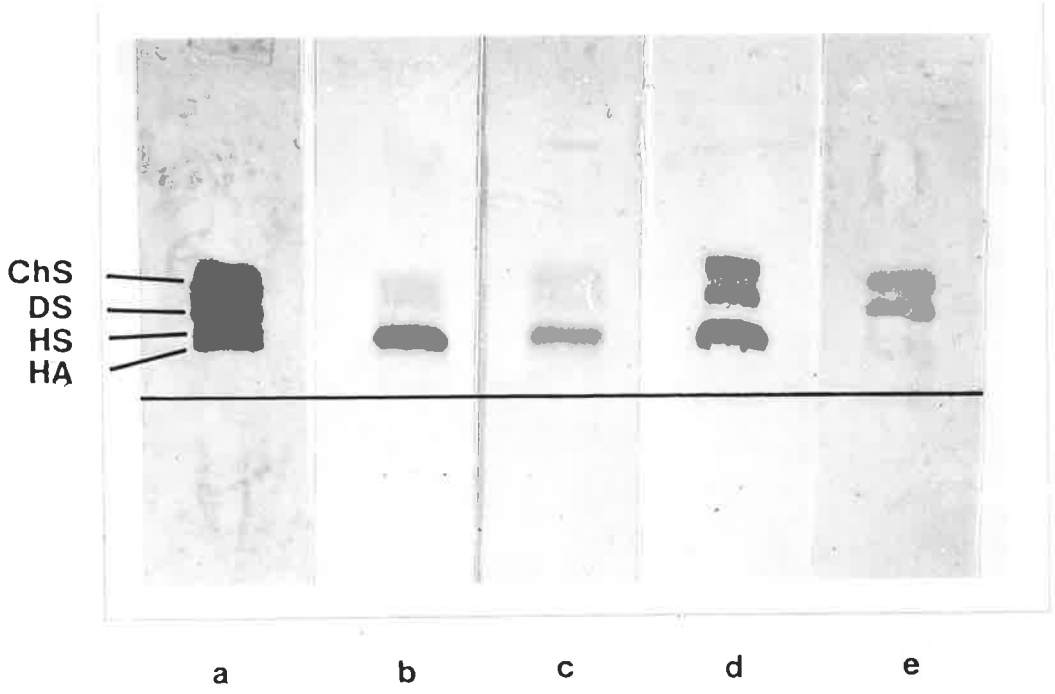


Figure 6.6

Electrophoretograms of glycosaminoglycans released by papain from the tissue residue.

(a) Standard glycosaminoglycans: hyaluronic acid (HA); heparan sulphate (HS); dermatan sulphate (DS); chondroitin sulphate 4 (ChS).

(b) Epithelial papain digest glycosaminoglycans.

(c) Epithelial papain digest glycosaminoglycans treated with Streptomyces hyaluronidase.

(d) Connective tissue papain digest glycosaminoglycans.

(e) Connective tissue papain digest glycosaminoglycans treated with Streptomyces hyaluronidase.

Samples (5 µl) of glycosaminoglycans were applied in a 0.5 cm line at the cathode and electrophoresed in 0.2 M calcium acetate, pH 7.2, at 30 volt/cm for 30 minutes. Stained with Alcian Blue.

Table 6.2

Proportions of Glycosaminoglycans Remaining in the Tissue after Extraction by 4 M Guanidinium Chloride.

Glycosaminoglycan	Proportions of Glycosaminoglycans Epithelium	Connective Tissue
Hyaluronic Acid	22.7%	35.9%
Heparan Sulphate	59.5%	10.2%
Dermatan Sulphate	8.9%	23.1%
Chondroitin Sulphate 4	8.9%	30.8%

The glycosaminoglycans released by papain digestion of the residual tissue following 4 M guanidinium chloride extraction were electrophoresed and their relative proportions determined by integration of densitometric scans. Values are expressed as percentages of the total glycosaminoglycans.

Table 6.3

Proportions of Glycosaminoglycans Constituting Proteoglycans Extracted by 4 M Guanidinium Chloride.

Glycosaminoglycans	Proportions of Glycosaminoglycans	
	Epithelium	Connective Tissue
Heparan Sulphate	80.0%	11.5%
Dermatan Sulphate	20.0%	50.0%
Chondroitin Sulphate 4	0	38.5%

The glycosaminoglycans were released from the proteoglycans extracted from the gingival epithelium and connective tissue in 4 M guanidinium chloride by papain and then electrophoresed. The relative proportions of each glycosaminoglycan species were determined by integration of densitometric scans of the electrophoresed glycosaminoglycans. Values are expressed as percentages of the total glycosaminoglycans.

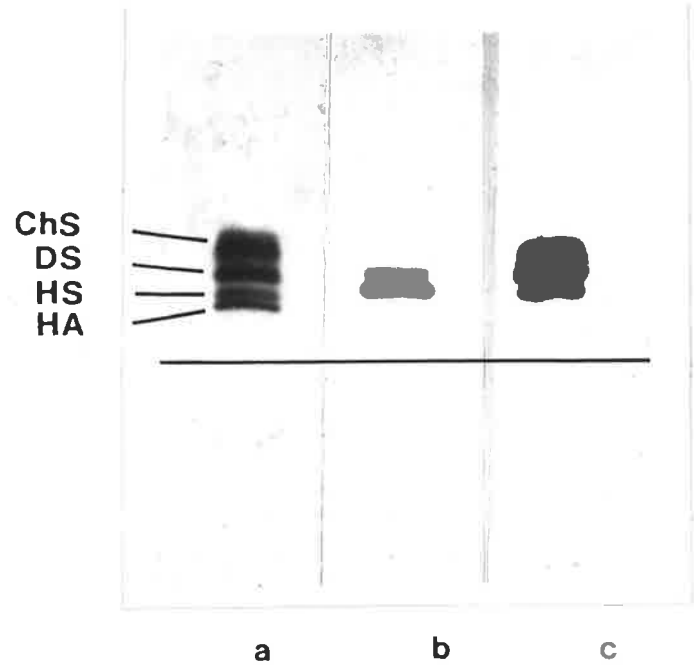


Figure 6.7

Electrophoretograms of papain digested gingival proteoglycans prepared for chromatography on Sepharose 4B-CL.

(a) Standard glycosaminoglycans: Hyaluronic acid (HA); heparan sulphate (HS); dermatan sulphate (DS) and chondroitin sulphate 4 (ChS).

(b) Epithelial proteoglycans.

(c) Connective tissue proteoglycans.

The glycosaminoglycans were obtained by papain digestion of an aqueously extracted sample of epithelial and connective tissue proteoglycans obtained from ion-exchange chromatagraphy and density gradient centrifugation. Electrophoretic conditions were the same as for Figure 6.6.

The glycosaminoglycan composition of each of the peaks obtained by column chromatography of the connective tissue proteoglycan fractions D_{CT} 1 and D_{CT} 2 on Sepharose 4B-CL were also analysed by electrophoresis following papain digestion (Figure 6.8). In each peak all three glycosaminoglycans, heparan sulphate, dermatan sulphate and chondroitin sluphate 4 were observed. However, there were some quantitative differences between each of these fractions noted (Table 6.4).

The largest connective tissue proteoglycan excluded from Sepharose 4B-CL under associative conditions, D_{CT} 1 (K_{av} 0.08), contained predominantly dermatan sulphate and chondroitin sulphate 4. The smaller molecular weight fractions, D_{CT} 1 (K_{av} 0.46) and D_{CT} 2 (K_{av} 0.31), contained dermatan sulphate as the predominant glycosaminoglycan with smaller proportions of heparan sulphate and chondroitin sulphate 4. The smallest molecular weight fraction, D_{CT} 2 (K_{av} 0.57), contained predominantly dermatan sulphate with very little heparan sulphate and chondroitin sulphate 4 being detected.

Amino Acid Analysis of Proteoglycan Core Protein

Amino acid profiles were obtained for protein cores of proteoglycans in the densest fraction of the density gradient preparations of gingival epithelium and connective tissue (D_E 1 and D_{CT} 1). The profiles obtained from such analyses are listed in Table 6.5. In both tissue preparations, glutamic acid was the major contributor for both epithelium and connective tissue. Aspartic acid and glycine were also major contributors to the overall amino acid compositions of these proteoglycans. Methionine was characteristically a minor component. Overall, the epithelial proteoglycans appeared to be characterized by a relatively higher serine and glutamic acid content,

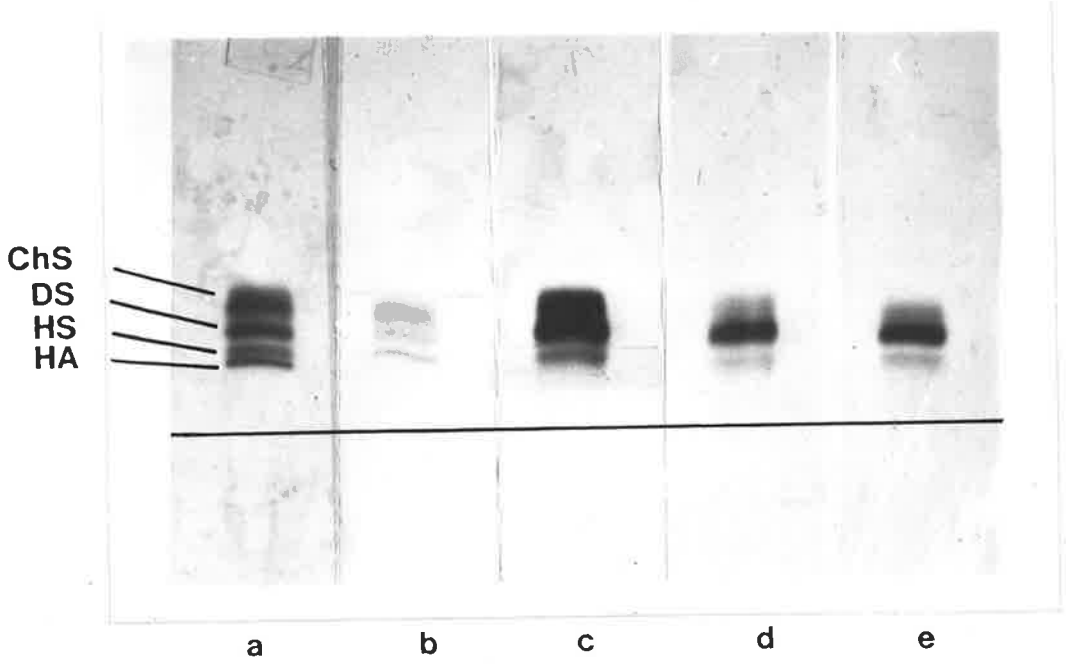


Figure 6.8

Electrophoretograms of the glycosaminoglycans of various connective tissue proteoglycans fractionated on Sepharose 4B-CL.

(a) Standard glycosaminoglycans: hyaluronic acid (HA); heparan sulphate (HS); dermatan sulphate (DS) and chondroitin sulphate 4 (ChS).

(b) Fraction D_{CT} 1; peak K_{av} 0.08.

(c) Fraction D_{CT} 1; peak K_{av} 0.46.

(d) Fraction D_{CT} 2; peak K_{av} 0.31.

(e) Fraction D_{CT} 2; peak K_{av} 0.57.

Following fractionation of the D_{CT} 1 and D_{CT} 2 fractions, aliquots from each peak were subjected to papain digestion and the glycosaminoglycans were then electrophoresed. The electrophoretic conditions were the same as for Figure 6.6.

Table 6.4

Proportions of Glycosaminoglycans Constituting the Connective Tissue Proteoglycans Fractionated on Sepharose 4B-CL.

Glycosaminoglycan	Proportions of Glycosaminoglycans in Chromatographic Peaks 1	2	3	4
Heparan Sulphate	32.0%	11.5%	14.8%	9.0%
Dermatan Sulphate	32.0%	50.0%	74.0%	82.5%
Chondroitin Sulphate 4	35.0%	38.5%	22.0%	8.5%

Chromatographic Peaks of Density Gradient Fractions

1. Fraction D_{CT} 1; peak K_{av} 0.08.
2. Fraction D_{CT} 1; peak K_{av} 0.46.
3. Fraction D_{CT} 2; peak K_{av} 0.31.
4. Fraction D_{CT} 2; peak K_{av} 0.57.

The glycosaminoglycans were released from the proteoglycans fractionated on Sepharose 4B-CL by papain and then electrophoresed. The relative proportions of each glycosaminoglycan species were determined by integration of densitometric scans of the electrophoresed glycosaminoglycans. Values are expressed as percentages of the total glycosaminoglycans in each sample.

Table 6.5

Amino Acid Composition of Gingival Epithelial and Connective Tissue Proteoglycans

	Residues per 1000 residues			
	Epithelium	Connective Tissue	Heparan Sulphate* Proteoglycan	Dermatan Sulphate† Proteoglycan
Asx	110	113	125	128
Thr	46	52	78	42
Ser	90	66	142	65
Glx	194	129	161	104
Pro	50	86	77	72
Gly	119	120	100	76
Ala	79	77	57	49
½Cys	ND	ND	ND	ND
Val	47	48	33	66
Met	3	trace	15	6
Ile	31	43	34	56
Leu	62	93	68	127
Tyr	21	20	19	23
Phe	27	27	16	29
Lys	53	63	41	87
His	10	11	12	23
Arg	38	52	21	32
Try	ND	ND	ND	ND

Proteoglycans from the D_E1 and $D_{CT}1$ fractions of the density gradient were analysed for amino acid composition following hydrolysis in 6 M HCl on a Beckman Model 120 C amino acid analyser. Values from the data of OLDBERG, et al. (1979) and DAMLE, et al. (1982) are included for comparison.

ND = Not determined

* Results from OLDBERG, et al, (1979).

† Results from DAMLE, et al, (1982).

whilst the connective tissue proteoglycan contained greater proportions of proline, leucine and arginine.

DISCUSSION

Proteoglycans have been isolated and partially characterized following extraction by guanidinium chloride from human gingival epithelium and connective tissue. The epithelial proteoglycans appeared to be comprised of only two glycosaminoglycans, namely, heparan sulphate and dermatan sulphate. This is surprising since chondroitin sulphate 4 is also a major contributor to the overall sulphated glycosaminoglycan composition of gingival epithelium. Indeed, previous studies (Chapter 3) indicate that chondroitin sulphate 4 contributes approximately 20% of the total epithelial glycosaminoglycans. In the results now being considered, the papain digestion of the residual epithelial tissue, following guanidinium chloride extraction, revealed chondroitin sulphate 4 together with hyaluronic acid, heparan sulphate and dermatan sulphate remaining in the tissue. The contribution by chondroitin sulphate 4 to the glycosaminoglycan content not extracted by guanidinium chloride was estimated to be 9%. Therefore, since approximately one-half of the total epithelial chondroitin sulphate 4 was extracted by guanidinium chloride it would appear that the extracted chondroitin sulphate 4 must have been lost during the purification steps of ion-exchange chromatography and density gradient centrifugation. This finding highlights the serious problem of recovery of these molecules when working with tissues such as gingival epithelium which contain extremely small quantities of proteoglycans.

Indeed, barely sufficient proteoglycans were extracted from human gingival epithelium to allow chromatography on Sepharose 4B-CL under associative conditions. As a result of the small quantities

of proteoglycans extractable from this tissue, only very small columns (29 cm x 0.6 cm) were used.

The profiles obtained from the chromatography of epithelial proteoglycans revealed a single, symmetrical peak which was completely included in the gel (K_{av} 0.53). This is in direct contrast to the previous studies on gingival proteoglycans (WIEBKIN, BARTOLD and THONARD, 1979; BARTOLD, WIEBKIN and THONARD, 1982) in which a small proportion of epithelial proteoglycans was demonstrated to be excluded from Sepharose 2B-CL. Such differences may be related to the different experimental procedures adopted. For example, in these studies, in vitro incorporation of [^{35}S]-sulphate into newly synthesized proteoglycans was measured. Under such conditions it appeared that a large molecular weight proteoglycan was synthesized which was excluded from Sepharose 2B-CL. Nonetheless, it is possible that very large proteoglycan species were present in small amounts in the preparations used in the present study. However, the method used to identify proteoglycans (a colourimetric uronic acid assay) may not have been sensitive enough to demonstrate such small quantities. Indeed, this is most likely, because the proportion of very large material excluded from Sepharose 2B-CL in the [^{35}S]-sulphate studies was never more than 20% of the total extracted proteoglycan. Furthermore, the sensitivity of [^{35}S]-sulphate as an indicator of proteoglycans is many times greater than the uronic acid assay used in this study which is only sensitive to a minimum of 3 µg uronic acid / ml.

The gingival connective tissue proteoglycans were easier to study because of the greater amounts present. Chromatography on Sepharose 4B-CL revealed at least three different proteoglycan species on the basis of molecular size. These corresponded to observed K_{av} values

on a column (69 cm x 0.9 cm) run under associative conditions of (a) 0.08; (b) 0.31 and 0.46 (c) 0.57. Glycosaminoglycan analysis of each of these three groups revealed a predominance of dermatan sulphate in the smaller sized proteoglycan fractions. For example, D_{CT} 1 proteoglycans of K_{av} 0.08 comprise almost equal proportions of heparan sulphate, dermatan sulphate and chondroitin sulphate 4, whilst D_{CT} 1 proteoglycans from K_{av} 0.31 and D_{CT} 2 proteoglycans with a K_{av} of 0.46 contain less heparan sulphate, proportionately more dermatan sulphate and slightly more chondroitin sulphate 4 than the D_{CT} 1 proteoglycans of K_{av} 0.08. The smallest proteoglycan fraction studied, D_{CT} 2 of K_{av} 0.57, contained predominantly dermatan sulphate, with heparan sulphate and chondroitin sulphate 4 being relatively minor contributors. These findings are of interest since they indicate a general polydispersity of gingival connective tissue proteoglycans not only with respect to molecular size but also glycosaminoglycan composition. Furthermore, they appear to be different from proteoglycans extracted from skin (DAMLE, et al., 1979) and periodontal ligament (PEARSON and GIBSON, 1982). The proteoglycans isolated by these workers are claimed to contain only chondroitin sulphate or only dermatan sulphate glycosaminoglycan side chains, in contrast to the proteoglycan fractions reported in the present study which appear to be comprised of more than one glycosaminoglycan species.

The previous observation that gingival connective tissue proteoglycans are not dissociated by 4 M guanidinium chloride (WIEBKIN, BARTOLD and THONARD, 1979) was confirmed by this study. The D_{CT} 1 fraction chromatographed into two populations (K_{av} 0.14 and 0.57) under dissociative conditions which were considered to be similar to the two populations observed under associative conditions

(K_{av} 0.08 and 0.31), since the proportion of larger sized material remained constant.

The amino acid profiles of both epithelial and connective tissue proteoglycans revealed some differences in their overall composition. However, with the exception of glutamic acid, these differences were small and at face value appear inconsequential. Nevertheless, in considering the amino acid composition of certain proteoglycans, some characteristics specific for gingival proteoglycan amino acid composition become apparent. For example, predominantly heparan sulphate proteoglycans isolated by OLDBERG, KJELLEN and HÖÖK (1979) appear to contain high proportions of aspartic acid, serine, glutamic acid and glycine. On the other hand, predominantly dermatan sulphate proteoglycans (DAMLE, CÖSTER and GREGORY, 1982) appear to have elevated amounts of aspartic acid, glutamic acid and leucine. Such differences in amino acid composition of different proteoglycans are highlighted in the gingival epithelial and connective tissue proteoglycans respectively of this study (Table 6.5). Epithelial proteoglycans, which appeared to be comprised of predominantly heparan sulphate, were higher in the amino acids aspartic acid, serine, glutamic acid and glycine, whilst the connective tissue proteoglycans, which contained greater amounts of dermatan sulphate, were higher in aspartic acid, glutamic acid, glycine and leucine amino acid residues.

In conclusion, this study, together with others from our laboratory (WIEBKIN, BARTOLD and THONARD, 1979; BARTOLD, WIEBKIN and THONARD, 1982), clearly demonstrates fundamental differences in the chemistry of proteoglycans extracted from human gingival epithelium and connective tissue with respect to composition and molecular size. The significance of these differences is not yet clear, although it is not unreasonable to speculate that the

extracellular matrices of these tissues possess different physico-chemical properties which are, perhaps, indicative of the tissue types. Indeed, the predominance of heparan sulphate rich proteoglycans in epithelium and dermatan sulphate rich proteoglycans in connective tissue, confirms the earlier observations that heparan sulphate and dermatan sulphate are characteristic glycosaminoglycans of gingival epithelium and connective tissue respectively, which reflect the general architecture of these two tissue types.

SUMMARY

Proteoglycans extracted from separated specimens of healthy human gingival epithelium and connective tissue have been purified. The epithelial proteoglycans fractionated as a single included peak on Sepharose 4B-CL and contained heparan sulphate and dermatan sulphate glycosaminoglycans. The connective tissue proteoglycans separated into three major populations on Sepharose 4B-CL. Of these populations, only the largest sized material was excluded under associative conditions (0.5 M sodium acetate, pH 7.4). Following subsequent fractionation of the excluded material under dissociative conditions (4 M guanidinium chloride in 0.05 M sodium acetate, pH 7.4) there appeared to be an absence of any aggregate formation of molecules within this population. The connective tissue proteoglycans contained heparan sulphate, dermatan sulphate and chondroitin sulphate 4, the proportions of which, varied with the molecular size of the proteoglycans. Amino acid analysis of the protein cores of gingival epithelial and connective tissue proteoglycans revealed differences which were similar to the differences described between other types of proteoglycans from various soft tissues.

CHAPTER 7

GENERAL DISCUSSION

To date, the chemistry of the non-fibrous components of the extracellular matrix of human gingivae (proteoglycans in particular) has recieved little attention compared with the fibrous components. Nonetheless, it is through this matrix, in which the connective tissue fibres are embedded and which also surrounds the epithelial cells, that nutrients and metabolic products must diffuse if these tissues are to exist in a healthy condition. Accordingly, the experiments described herein were designed to obtain a better understanding of the chemical nature of the proteoglycans of the extracellular matrix of healthy human gingivae. In particular, the macromolecular, uronate containing components of the epithelial and connective tissue extracellular compartments were to be compared. The experimental protocol by which this was to be achieved was bilateral. Firstly, the principal components of the proteoglycans, (glycosaminoglycans and protein) were to be isolated and characterized. Following this, intact proteoglycan molecules were to be investigated.

Whilst characterization of the extracellular matrix components of healthy gingival epithelium and connective tissue is essential for understanding the characteristics of diseased gingiva (such as that seen in periodontal disease) there are other reasons for studying gingival tissue. For example, gingival tissue could well be the tissue of choice for studying inflammation, since the biochemistry of chronic marginal periodontitis parallels the biochemistry of chronic inflammation in many ways.

Chapter 3 describes studies regarding the isolation, identification and quantitation of the glycosaminoglycan species within gingival epithelium and connective tissue. Whilst there were no qualitative differences noted between the glycosaminoglycans of epithelium and connective tissue, important quantitative differences were noted for the

molecules between these two tissues. Gingival epithelial glycosaminoglycans contributed much less to the overall weight of this tissue (0.07%) than did the glycosaminoglycans of the connective tissue (0.23%). Furthermore, quantitative differences were noted for individual glycosaminoglycan species within the epithelium and connective tissue. Heparan sulphate was the predominant glycosaminoglycan of epithelium whilst dermatan sulphate was the major contributor to the glycosaminoglycan species of connective tissue. The functional associations of heparan sulphate with cell surfaces and dermatan sulphate with mature collagen were alluded to in Chapter 3 as being possible explanations for the predominance of heparan sulphate in the epithelium and dermatan sulphate in the connective tissue. Nevertheless, hyaluronic acid and chondroitin sulphate are also present, and cannot be overlooked as important contributors to the extracellular matrices of gingiva, since they too, presumably serve functional roles. Such roles which have been ascribed to these two glycosaminoglycans are related to cellular growth, cell mobility and control of biosynthetic functions (WIEBKIN and MUIR, 1973 a & b; DIETRICH, et al., 1977; CHIARUGI and DIETRICH, 1977). Therefore, the various glycosaminoglycans of gingiva may conceivably fulfil diverse functions.

The gingival glycosaminoglycan molecular weight studies described in Chapters 4 and 5 have highlighted some interesting points. Not only do they provide information regarding the molecular sizes of these molecules, but they also describe useful refinements of techniques used in the study of glycosaminoglycans. Indeed, the techniques adopted to estimate the molecular weights of the sulphated gingival glycosaminoglycans should be of use to others studying tissues

such as gingivae which are limited in their availability for analysis and do not contain large proportions of glycosaminoglycans. In this regard, a simple, rapid, monodimensional electrophoresis system was developed which permitted improved separation of the glycosaminoglycans extracted from gingiva. By utilizing the technique of end group labelling, as originally described by ROBINSON and HOPWOOD (1973) a simple, but accurate, method of number-average molecular weight estimation has been developed for very small amounts of sulphated glycosaminoglycans. Consequentially, separation of glycosaminoglycans by the classical techniques of ion-exchange and gel chromatography, which have doubtful resolution capacity (see Chapter 4) and require relatively large quantities of glycosaminoglycans, has been avoided. Quantities as low as a few micrograms may be analysed for molecular weight using the above technique.

The number-average molecular weights estimated for the sulphated gingival glycosaminoglycans ranged from 12,300 for heparan sulphate in the epithelium to 27,000 for dermatan sulphate, also from the epithelium. There was little variation of molecular weights for similar glycosaminoglycan species isolated from the two tissues.

The data from the ultracentrifuge studies of hyaluronic acid extracted from gingival epithelium and connective tissue described in Chapter 5 were the most difficult to interpret of the experiments described in this thesis. Indeed, the only way in which an intelligable interpretation of the data could be made was after extensive statistical analysis had been carried out. In doing so, we were able to predict, within certain confidence limits, the $(s^{-1})_o$ value for various hyaluronic acid preparations. Following statistical prediction of $(s^{-1})_o$ values, molecular weight values could be estimated. On the basis of these studies, hyaluronic acid isolated from human gingival epithelium was observed to be different from that isolated

from the connective tissue. For example, the sedimentation behaviour of epithelial hyaluronic acid in the analytical ultracentrifuge appeared to be influenced to a greater degree by its concentration than the connective tissue hyaluronic acid. Furthermore, the statistically derived values of $(s^{-1})_o$ indicated epithelial hyaluronic acid could be possibly two times larger with respect to molecular weight.

The findings concerning hyaluronic acid are in direct contrast to those reported for the sulphated glycosaminoglycans from gingiva which showed little inter-tissue molecular weight variability. Whether or not the different molecular weights of hyaluronic acid are related to different functional properties of this macromolecule in epithelium and connective tissue remains to be established.

The intact proteoglycans extracted from gingival epithelium and connective tissue under dissociative conditions were studied and described in Chapter 6. This study was confined to observing the gel chromatographic profiles of the proteoglycans, the glycosaminoglycan composition of these proteoglycans and the amino acid composition of these macromolecules. From the data obtained and that reported previously (WIEBKIN, BARTOLD and THONARD, 1979; BARTOLD, WIEBKIN and THONARD, 1982), it is clear that the proteoglycans of gingival epithelium and connective tissue are different with respect to molecular size and composition. The epithelial proteoglycans were comprised of predominantly heparan sulphate and a quantitatively minor component of dermatan sulphate, whilst the connective tissue proteoglycans contained heparan sulphate, dermatan sulphate and chondroitin sulphate 4 with dermatan sulphate predominating quantitatively. These findings substantiate the earlier observations reported in Chapter 3 that heparan sulphate is a major component of the epithelial glycosaminoglycans whilst the connective tissue

glycosaminoglycans contain dermatan sulphate in the greatest proportion.

The results reported in the experimental chapters have, therefore, highlighted several quantitative and qualitative differences between the proteoglycans of human gingival epithelium and of connective tissue. These findings are not dissimilar to those reported for skin, a tissue which is structurally quite similar to gingiva, being comprised of a stratified squamous epithelium and an underlying connective tissue.

Chemical analyses of digests of both human and animal skin have demonstrated that the dermis contains more glycosaminoglycans per milligram of dry tissue than the epidermis. Indeed, MIER and WOOD (1969) reported the amount of glycosaminoglycans in human epidermis was only one-fifth of that in the dermis. A similar distribution of glycosaminoglycans between gingival epithelium and connective tissue is reported in Chapter 3.

The individual glycosaminoglycan species comprising the extracellular matrices of skin have been studied by various means and conflicting reports concerning their identification are prevalent in many of the early reports (BARKER, CRUICKSHANK and WEBB, 1965; MIER and WOOD, 1969; BARKER, KENNEDY and SOMERS, 1969). More recently, the glycosaminoglycans hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sluphate 4 and/or chondroitin sulphate 6 have been demonstrated to be constituent species of mammalian skin (KING and TABIOWO, 1980; KING, 1981). Characterization by KING (1981) of epidermal glycosaminoglycans synthesized in organ culture indicated hyaluronic acid and heparan sulphate to be the major glycosaminoglycan species of this tissue. On the other hand, TAJIMA and NAGAI (1980) found that dermatan sulphate predominates within the extracellular matrix of skin dermis.

As for gingival proteoglycans, the proteoglycans of skin have not been characterized as comprehensively as the glycosaminoglycans which constitute the carbohydrate moiety of these macromolecules. Indeed, no reports have been found concerning the proteoglycan composition of epidermis and dermis separately. Nonetheless, several recent studies have characterized two major proteoglycan species obtained from extracts of porcine and bovine skin (ÖBRINK, 1972; DAMLE, et al., 1979; DAMLE, CÖSTER and GREGORY, 1982; PEARSON and GIBSON, 1982).

These two proteoglycans are primarily comprised of either dermatan sulphate or chondroitin sulphate bound to a protein core via alkali labile linkages; they were polydisperse with respect to molecular weight and size and demonstrate no capacity to form aggregates with hyaluronic acid. Whilst these findings are not identical to those reported for gingival proteoglycans (Chapter 6), similarities are evident, especially with respect to polydispersity of molecular size and the absence of interactivity of connective tissue proteoglycans with hyaluronic acid.

The significance of the characterization of gingival proteoglycans reported in this study becomes relevant when considering the pathogenesis of human periodontal disease. Histological observations on periodontally diseased gingival tissue have indicated a breakdown of the connective tissues and changes in the integrity of the epithelium (STAHL, SANDLER and SUBEN, 1958; QUINTARELLI, 1960 a & b; MELCHER, 1967). Refer to Figure 7.1 for an anatomical description of the periodontium.

Such changes are a result of the accumulation of bacterial plaque on teeth (LÖE, THIELADE and JENSEN, 1965) and were originally considered to progress via the sulcular epithelium to the connective tissue

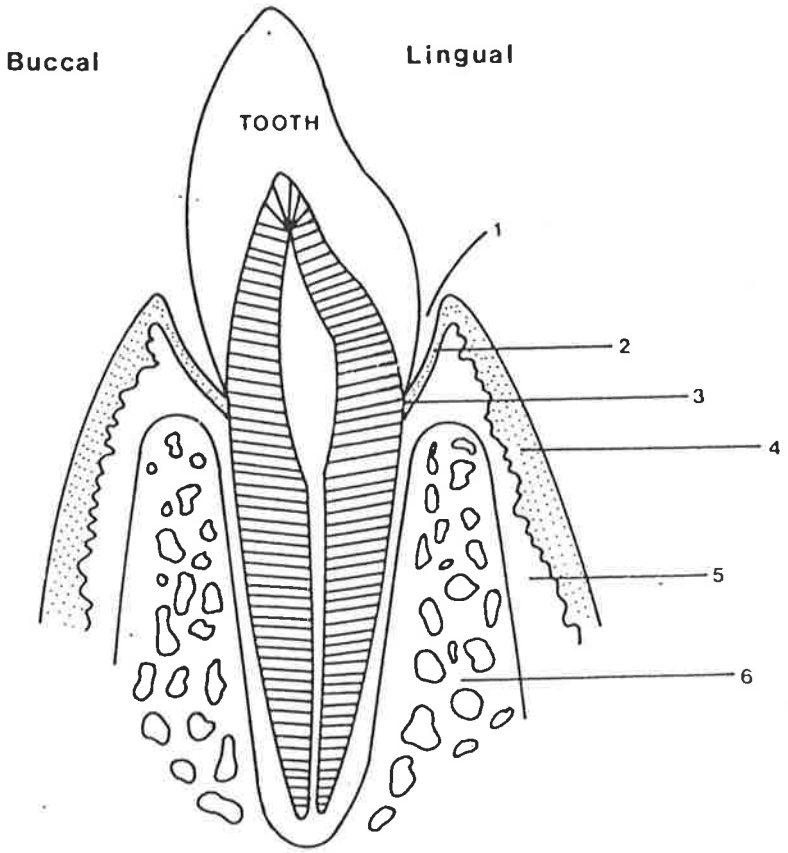


Figure 7.1

Anatomy of the periodontium. Diagram of a vertical section through a tooth and its supporting structures.

1. Gingival Sulcus
2. Sulcular Epithelium
3. Junctional Epithelium
4. Oral Epithelium
5. Connective Tissue
6. Alveolar Bone

because "...*the sulcular epithelium is thin and not keratinized. It forms, therefore, per se, no formidable barrier against penetration by bacterial products or invasion of bacteria*" (SCHULTZ-HAUDT and LUNDQVIST, 1962). However, whilst the above observations imply that there is a breach of the epithelium before the progressing periodontal lesion reaches the connective tissue, this has never been conclusively shown to be the case. In fact, THILANDER (1968) demonstrated that during the early stages of gingivitis, the histological appearance of the sulcular epithelium was quite normal with an intact basement membrane, whilst at the same stage, changes were observed in the underlying connective tissue in the form of dilated blood vessels and slight oedema. McDOUGALL (1971, 1972) was amongst the first to clearly demonstrate that the initiating stimuli (irritants) for periodontal disease arose in the gingival sulcus and permeated to the connective tissue via the junctional epithelium and not the sulcular epithelium. Indeed, the epithelium remains relatively intact during the early stages of periodontal disease. Hence, despite the enormous irritation the sulcular and junctional epithelium encounter from the bacterial plaque which colonizes the gingival sulcus, the physicochemical and biochemical nature of the epithelial extracellular matrix, its susceptibility to degradation and its capacity to regulate diffusion of molecules, could be important in determining the early stages of periodontal disease.

The importance of the proteoglycans in maintaining tissue integrity in a tissue such as gingiva can only be speculatory at this stage. Nonetheless, the implications of the present findings to the factors involved in the progression of periodontal disease are of interest. For example, it is evident that there are two

large macromolecular populations, namely hyaluronic acid and the proteoglycans in the extracellular matrix of gingival epithelium. In this regard, it is noteworthy that since the actual space available in the epithelium for occupancy by such macromolecules is very small, and complex macromolecular arrangements have previously been reported (WIEBKIN, BARTOLD and THONARD, 1979), one could expect the effective concentration of these molecules to be high. Conversely, the extracellular "space" available within the connective tissue is greater and it is easier to visualize how such large molecules might occupy these regions. Furthermore, the recent findings that heparan sulphate chains may interact to form aggregates (FRANSSON, NIEDUSZYNSKI and SHEEHAN, 1980), further implicate the possibility of complex macromolecular interactions and arrangements within the gingival epithelium. Such putative molecular interactions of the epithelial extracellular matrix may act as a "first-line" defence mechanism by specifically regulating molecular penetration of the junctional and the sulcular epithelia.

Therefore, the question arises as to how the effecting stimuli penetrate the connective tissue to bring about an inflammatory and immune response without first disrupting the epithelium? One possible explanation based on the results of this study is outlined below.

The inflammatory reaction reported to occur in gingival connective tissue affected by periodontal disease is considered to arise from irritants reaching it from the gingival plaque. Such irritants may either cause tissue destruction in their own right (e.g. enzymes) or act as antigens, so resulting in an immunological response which may subsequently cause tissue damage. Two major groups of irritants have therefore been implicated in such processes; a) hydrolytic enzymes, and b) bacterial endotoxins. It is of important

note that bacteria have not been shown to invade gingival epithelium during the early stages of periodontal disease.

Despite evidence that substrates for the various hydrolytic enzymes produced by oral bacteria are present in gingival epithelium and connective tissue, pretreatment of gingival tissue both *in vitro* and *in vivo* does not cause complete disaggregation of the extracellular matrices (SCHULTZ-HAUDT, 1955, THONARD, 1960). This is, indeed, not surprising, since in the light of current findings, such enzymes are no longer considered to play an important role in the early stages of proteoglycan degradation (see Chapter 1, section on Proteoglycan Degradation, page 48). However, whilst degradation due to hydrolytic enzymes may be minimal, these enzymes, if they were to reach the connective tissue, would act as antigens and induce an immunologically mediated inflammatory response.

Endotoxins (lipopolysaccharides in particular) have also been studied regarding their potential as initiators of periodontal disease (MERGENHAGEN, 1967, MERGENHAGEN, TEMPEL and SNYDERMAN, 1970). Electronmicroscopic studies have demonstrated gingival bacterial lipopolysaccharide to be either circular shaped (diameter 250-400 Å) or rod-like (90-130 Å in width and 250-400 Å in length) (SELVIG, HOFSTAD and KRISTOFFERSON, 1971). Furthermore, these endotoxins are antigenic and are, therefore, capable of inducing an immunological reaction within gingival connective tissues (HOFSTAD, 1970).

Such macromolecules would conceivably be limited in their capacity to diffuse through the concentrated proteoglycan rich extracellular matrix of intact gingival epithelium. In this regard, the concept of epithelial permeability must be considered and would seem to be of paramount importance with respect to the initiation of periodontal disease. Indeed, gingival epithelium is permeable

to both the passage of substances from the connective tissue into the oral cavity as well as the passage of materials from the oral environment into the gingival connective tissue.

Evidence for an outward flow of substances (gingival crevicular fluid) from the gingival connective tissue into the oral cavity is well established and has been reviewed by CIMANSONI (1974). In gingival health there is a small, but detectable, outward flow of gingival crevicular fluid which increases as periodontal disease becomes established. Principally, this fluid represents an inflammatory exudate. In addition, leukocytes may also enter the oral cavity from the connective tissue via the gingival sulcus (WRIGHT, 1964). The most likely route for the transport of both gingival crevicular fluid and leukocytes is the intercellular "spaces" of the junctional epithelium (McDOUGALL, 1970; SCHROEDER, 1970). Furthermore, it has been concluded that the outward flow of gingival crevicular fluid is probably the result of the hydrostatic and osmotic parameters which control extravascular fluids (CIMANSONI, 1974).

However, of more importance to the pathogenesis of periodontal disease, is the ingress of materials from the oral cavity to the gingival connective tissue against the outward flow of gingival crevicular fluid. TOLO (1971), using tritiated albumin, demonstrated that this protein, of molecular weight 68,000 could diffuse through the sulcular epithelium of experimental animals. This was in contrast to the findings of GIBSON and SHANNON (1965), who failed to observe any diffusion of carbon particles through gingival epithelium. However, TOLO (1971) explained the difference on the basis of particle size and shape and concluded that albumin was of such a shape that it could penetrate the extracellular matrix of gingival sulcular epithelium more readily than carbon particles. Similar findings

of diffusion through intact sulcular epithelium have been demonstrated using E. coli endotoxin (SCHWARTZ, STINSON and PARKER, 1972). McDOUGALL (1971) demonstrated that the penetration of horseradish peroxidase (molecular weight 40,000) through the extracellular matrix of sulcular epithelium occurred rapidly, reaching the most apical cells of the junctional epithelium within ten minutes. Associated with this diffusion, was an observable widening of the intercellular "spaces". The marker protein was reported to eventually pass through the basement membrane into the underlying connective tissue and this was associated with dilation of venules, emigration of neutrophils into the junctional epithelium and ultimately macrophage and fibroblast uptake of the horseradish peroxidase.

The findings of McDOUGALL (1971) on the rapid penetration of a relatively large protein through an intact epithelial extracellular matrix are, at first sight, perplexing. Indeed, one would expect such macromolecules to be hindered in their movement through these matrices. However, based on the recent observations of CUMMING, HANDLEY and PRESTON (1979), that the diffusion of flexible macromolecules may, infact, be enhanced through concentrated polysaccharide matrices (presumably by molecular exclusion effects) an argument could be advanced for a similar diffusion mechanism governing the passage of flexible enzymes or endotoxins through the extracellular matrix of gingival sulcular epithelium. If this were to be the case, then such a model could explain, in part, the observations of connective tissue changes occurring before epithelial alterations during the pathogenesis of periodontal disease.

A further consideration regarding the question of how periodontal disease progresses is the possible biosynthetic regulatory effect of connective tissue on epithelium. For example, despite previous

demonstrations that gingival epithelium is capable of synthesizing its own extracellular proteoglycans in vitro (WIEBKIN, BARTOLD and THONARD, 1979; WIEBKIN and THONARD, 1981, 1982), proteoglycan synthesis by skin epidermis in vitro, is maximal when the dermis is present (KING and TABIOWO, 1980). These workers therefore postulated that the dermis may provide a necessary physical substratum for the epidermis, or, provide an essential co-factor or metabolic intermediate for optimal epidermal cell metabolism. This could be of relevance to gingival tissue in which the connective tissue extracellular matrix may be disrupted due to immunological and inflammatory reactions occurring within it. Such disaggregation of the gingival connective tissue matrix could conceivably have ramifications for the epithelium with respect to proteoglycan synthesis and therefore lead to the eventual epithelial breakdown as seen in the more advanced stages of periodontal disease.

To propose such mechanisms of enhanced diffusion of antigens or metabolic regulatory effects of connective tissue on epithelium requires, however, considerable extrapolation to an in vivo disease situation. Nonetheless there are other mechanisms which may equally account for gingival tissue destruction. For example, the release of proteoglycan degrading enzymes, in contrast to glycosaminoglycan degrading enzymes which are not considered to be responsible for the initial stages of proteoglycan degradation (see Chapter 1, section on Proteoglycan Degradation, page 48), by bacteria in gingival plaque has not yet been established and the relevance of proteinases released by the host cells is only beginning to be realized. Such possibilities remain to be investigated.

Indeed, the work described in this thesis has opened a variety of avenues for future investigation before the precise nature of the proteoglycans of human gingival epithelium and connective tissue as well as their contribution to gingival tissue integrity can be determined. For example, the chemical composition of both heparan sulphate and dermatan sulphate should be investigated. In particular, the relationship between iduronic acid content and self association properties of these two glycosaminoglycans (see Chapter 1, section on Proteoglycan Interactions, page 35) should be investigated in the light of the self associating properties of epithelial proteoglycans previously demonstrated (WIEBKIN, BARTOLD and THONARD, 1979). Furthermore, the interactive capacity of epithelial proteoglycans with epithelial hyaluronic acid as well as a more precise analysis of hyaluronic acid molecular weight should be attempted. Such additional information should provide a clearer picture of the gingival extracellular matrices and their various macromolecular interactions.

In addition to characterization of healthy gingival proteoglycans, gingivae affected overtly by periodontal disease must be characterized. In particular, studies concerning the products of gingival proteoglycan degradation as a result of chronic inflammation will give some insight into the nature of the causative degradative agents. As a result, investigations involving the isolation and identification of specific destructive gingival enzymes, both with respect to their source and mode of action, would be logical progressions.

A further possibility to be considered is the effect of chronic inflammatory periodontal disease on extracellular macromolecular interactions. Such interactions in healthy tissues include those between proteoglycans and collagen, hyaluronic acid, cell surface

components and other proteoglycans; all of which contribute to the overall extracellular network. Therefore, the question must be asked, if the proteoglycans were altered during chronic inflammation, what would be the effect on the above mentioned interactions and the whole matrix as a functional unit?

In conclusion, the findings obtained from the present study, together with those present in the literature, clearly indicate that future work in the field of gingival proteoglycan chemistry must be carried out systematically. There is a need for further data concerning the composition of the extracellular matrix of normal, healthy gingival epithelium and connective tissue. Indeed, only until the chemistry of healthy tissues is understood, and not before, can the process of destructive diseases begin to be elucidated and fully appreciated.

APPENDICES

APPENDIX 1

GINGIVAL TISSUE TRANSPORT SOLUTION

	gm/litre
EDTA	0.372
NaCl	6.83
KCl	0.2
Na_2HPO_4	1.15
KH_2PO_4	0.2
Phenol Red	0.001

The solution was adjusted to pH 7.2 with 10 M NaOH, and stored at $4^{o}C$.

APPENDIX 2

HISTOLOGICAL METHODS

Fixatives:

Neutral Formal-saline

Formalin (40% formaldehyde)	10 ml
NaCl	0.9 gm
Distilled Water	to 100 ml

Solution buffered to pH 7.0 by adding 3.5 gm of anhydrous NaH_2PO_4.

Stains:

a) Ehrlich's Haematoxylin

Haematoxylin	5 gm
Distilled Water	700 ml
Glycerol	300 ml
Ammonium Alum	50 gm
Sodium Iodate	0.2-0.4 gm
Acetic Acid (glacial)	20 ml

b) Eosin

95% ethanol	390 ml
1% eosin	50 ml
Acetic Acid (glacial)	2 ml
Phloxine	5 ml

APPENDIX 3

SOLUTION FOR EPITHELIUM AND CONNECTIVE TISSUE SEPARATION

	gm/litre
EDTA	3.723
NaCl	6.83
KCl	0.2
Na_2HPO_4	1.15
KH_2PO_4	0.2
Phenol Red	0.001

The solution was adjusted to pH 7.2 with 10 M NaOH and stored at $4^{o}C$.

APPENDIX 4

CONFIRMATION OF GINGIVAL GLYCOSAMINOGLYCAN SPECIES

The glycosaminoglycans extracted from human gingival epithelium and connective tissue were independently identified by Dr. J.J. Hopwood (Adelaide Children's Hospital) using a newly developed electrophoretic system (HOPWOOD and HARRISON, 1982). In this system dermatan sulphate electrophoreses as two bands, dermatan sulphate I and dermatan sulphate II. The dermatan sulphate II band has the same mobility as hyaluronic acid and therefore the values listed under dermatan sulphate II/hyaluronic acid are combined values for both hyaluronic acid and dermatan sulphate II. Nonetheless, hyaluronic acid is presumed to be present on the basis of the findings of Chapter 3.

The values listed in the ensuing Table A4.1 are percentages of the total glycosaminoglycan composition of human gingival epithelium and connective tissue respectively.

Table A4.1

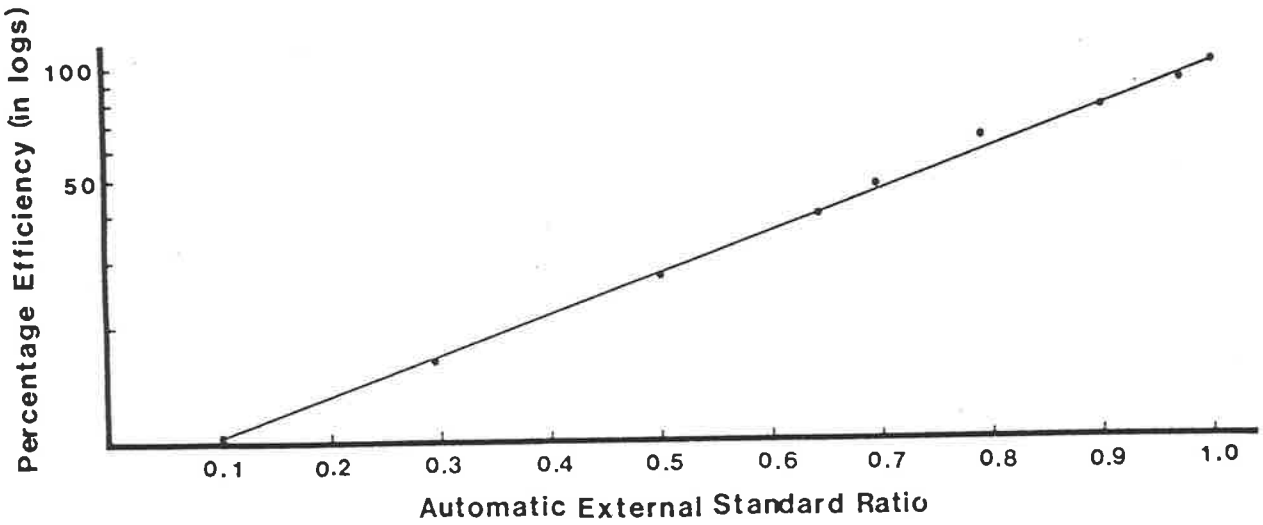
PROPORTIONS OF GINGIVAL GLYCOSAMINOGLYCANS

Glycosaminoglycan	Epithelium	Connective Tissue
Dermatan Sulphate I	7%	22%
Heparan Sulphate	53%	10%
Hyaluronic Acid/Dermatan Sulphate II	18%	38%
Chondroitin Sulphate 4	22%	30%
Chondroitin Sulphate 6	None detected	None detected
Keratan Sulphate	None detected	None detected

These values confirm the observation of heparan sulphate in both gingival epithelium and connective tissue glycosaminoglycan samples (Chapter 3) as well as confirm that heparan sulphate is the predominant glycosaminoglycan of epithelium whilst dermatan sulphate is the major contributor to the connective tissue glycosaminoglycans. In both tissue samples neither chondroitin sulphate 6 nor keratan sulphate were observed.

APPENDIX 5

LIQUID SCINTILLATION QUENCH CORRECTION CURVE



Using the automatic external standard of a Packard Tri-Carb liquid scintillation counter (Model 2405) and the channels ratio method with standard quenched samples of tritium, a quench correction curve for tritium was obtained. These standards were supplied by the Packard Instrument Company, Downers Grove, Illinois, U.S.A. and contained nitromethane as the quenching agent. The efficiency of the liquid scintillator in counting tritium had previously been determined as 50.65% using a standard unquenched tritium sample.

APPENDIX 6

GLYCOSAMINOGLYCAN REFERENCE STANDARDS

Glycosaminoglycan standards were kindly donated by Dr. M.B. Mathews, Department of Pediatrics, University of Chicago, U.S.A. Essential chemical and physical data together with descriptions of the methods of preparation were supplied. The following are verbatim extracts from this information bulletin which are relevant to the glycosaminoglycan standards used in the present study.

Hyaluronic Acid

Human umbilical cords were digested with papain and a crude hyaluronic acid fraction obtained by cetylpyridinium chloride precipitation. A purified hyaluronic acid preparation was obtained by fractionaal precipitation with ethyl alcohol of sodium chloride solutions of the crude fraction (1).

Chondroitin Sulphate 4

This preparation was obtained after succesive cetylpyridinium chloride precipitation, alcohol fractionation and fractional elution from an anion exchange resin of a papain digest of notochord of the river sturgeon, S. platorhyncus (1). Analysis (2) by digestion with with Proteus vulgaris enzymes revealed a composition of 74% 6-sulphated disaccharide, 15% 4-sulphated disaccharide and 11% unsulphated disaccharide.

Dermatan Sulphate

Crude by-products remaining after the isolation of heparin from hog mucosal tissues was obtained as a gift from Dr. H.H.R. Weber of Wilson Laboratories, Chicago. Dermatan sulphate was isolated as the copper complex, and purified by fractional elution from an anion exchange resin and by fractional precipitation with

alcohol (1,3). Analyses (2) by Proteus vulgaris enzymes showed 68% 4-sulphated disaccharide, 14% 4- 6- disulphated disaccharide, 7% 6-sulphated disaccharide, 1% unsulphated disaccharide and 10% chondroitinase ABC-resistant hexosamine derivitives. Of the total uronic acid content, 96% is iduronic acid and 4% is glucuronic acid (4).

Heparan Sulphate

By products from the preparation of heparin from beef lung were obtained as a gift from Dr. L.L. Coleman of Upjohn Laboratories, Kalamazoo, Michigan. The 1.25 M sodium chloride eluate from an anion exchange resin column was treated with trichloroacetic acid to remove nucleic acid contaminants and subjected to fractional precipitation with ethanol, N-sulphation (5) and gel filtration. Of the total uronic acid present, 33% is iduronic acid, 67% is glucuronic acid. The molar ratio of N-sulphate to glucosamine is 0.41 (4). The preparation showed an anticoagulant activity of 2.3 International Units per milligram.

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Table A6.1

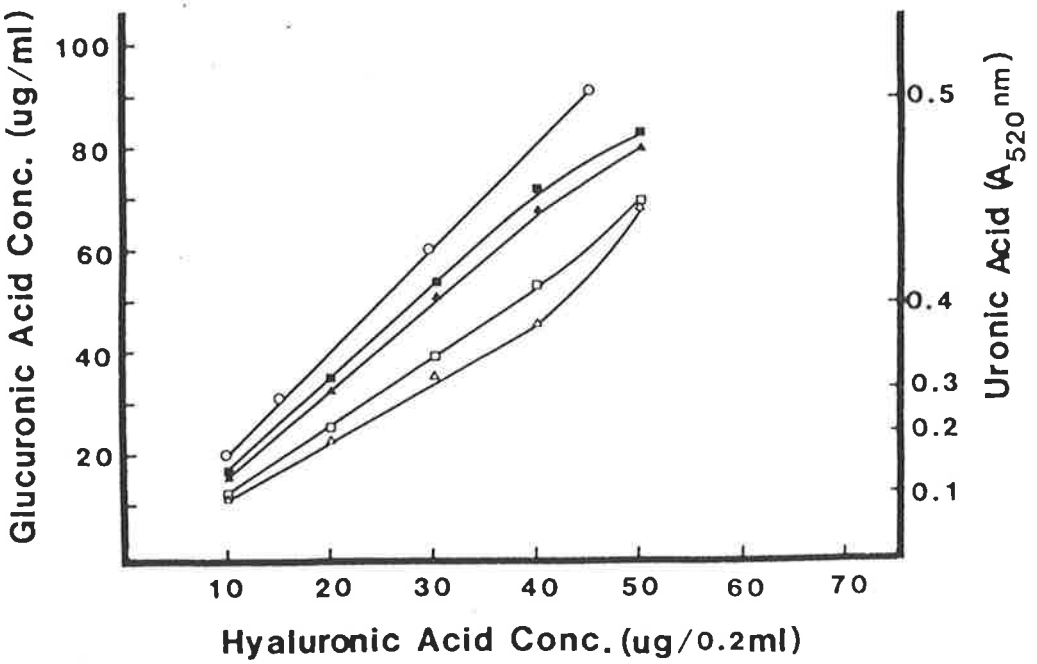
Analytical and Physical Data for Sodium Salts of Glycosaminoglycans

	HA	ChS 6	ChS 4	DS	HS
Nitrogen[1]	3.0	2.9	3.1	2.6	2.7
Hexuronic Acid[1]	47.2	34.8	32.0	15.5	49.2
Hexosamine[1]	38.3	31.4	27.9	31.5	28.2
Sulphate[2]	0.0	0.9	0.98	1.13	0.97
Xylose[1]	trace	0.4	1.0	0.1	0.2
Galactose[1]	trace	0.9	2.4	0.3	0.6
Galactosamine[3]	0.001	0.001	1.0	1.0	trace
Glucosamine[4]	1.0	trace	trace	0.015	1.0
Aspartic Acid[5]	0.001	0.007	0.024	trace	0.006
Serine[5]	0.001	0.021	0.6	trace	0.02
Threonine[5]	trace	0.004	0.007	trace	0.004
Glutamic Acid[5]	0.001	0.005	0.021	trace	0.006
Glycine[5]	0.001	0.013	0.053	trace	0.018
Intrinsic Viscosity[6]	540	116	38	84	57
Molecular Weight[7]	230,000	29,000	15,000	45,000	....

1. Percent by weight
2. Molar ratio of ester sulphate to hexosamine
3. Molar ratio to glucosamine
4. Molar ratio to galactosamine
5. Amino acid ratios corrected only for loss of hexosamine upon hydrolysis
6. ml/gm
7. Viscosity average molecular weight

APPENDIX 7

RELATIONSHIP BETWEEN COLOUR ABSORPTION AND CONCENTRATION OF GLYCOSAMINOGLYCANS



Using the method of Blumenkrantz and Asboe-Hansen (Anal. Biochem. 54, 484-489, 1973) the relationship between colour absorption and concentration of individual glycosaminoglycans and D-glucuronic acid was determined.

- ○—○ Hyaluronic Acid
- ■—■ Heparan Sulphate
- ▲—▲ Dermatan Sulphate
- △—△ Chondroitin Sulphate 4
- □—□ Chondroitin Sulphate 6

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